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# THE INFLUENCE OF COOKED SAUSAGE WITH INULIN ON THE PHYSIOLOGICAL INDICATORS OF LABORATORY ANIMALS

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**ABSTRACT**

Reducing fat content in meat products requires a complex of studies on the quality of sausage with fat substitutes and their impact on health. The article presents the study results concerning the effect of cooked sausage with inulin on the clinical and physiological parameters of laboratory animals. The experiment was performed for 26 days on 27 Wistar rats. The animals in the experimental group (Group 1) consumed cooked sausage with inulin. The control group (Group 2) was fed with cooked sausage with fat content of 20.5 ± 2.1%. Animals in the intact group (Group 3) consumed a standard diet of vivarium consisting of pearl barley porridge and compound feed. Animals in Group 1 had a minimum weight gain for 26 days of 8.6% compared to Group 2 (10.5%) and intact animals (13.0%). Group 1 rats showed a more pronounced rise in glucose levels with increase in total bilirubin and urea, decrease in creatinine, and increase in aspartate aminotransferase and alanine aminotransferase levels as compared to blood serum of animals in Group 2. Based on a decrease in total protein with increased total bilirubin, urea and glucose in Group 1 animals, the assumption was made of accelerating the catabolism of proteins and carbohydrates by the introduction of cooked sausage with reduced caloric content into the diet. It was established that the introduction of experimental meat products into the diet allows to reduce the total cholesterol, triglycerides and low-density lipoproteins in the blood and increase the level of high-density lipoproteins and lipase.

## 1. Introduction

In developed countries, the problem of high fat consumption is very topical. The population is concerned about the increase in mortality from cardiovascular and oncological diseases, and growing obesity. One of the measures aimed at solving this problem is to increase the production of low-calorie products. Many countries develop national programs to improve the health of the population, including measures to reduce the fat content in food. Fat has a wide range of technological properties that allow to create consumer characteristics of meat product, including texture, color, flavor, and taste. The fat content may be reduced either by replacing it by meat with a high content of muscle and connective tissue or by using non-meat ingredients with a structure different from fat (proteins, polysaccharides, and their mixtures). It should be noted that increasing the proportion of lean meat in meat product formulation may lead to unsatisfactory consumer characteristics, especially hard texture. In this regard, the selection of non-

meat ingredients that can simulate fat in the product is of particular interest. Development of fat substitutes is being carried out in many countries, including Russia. For this purpose, a diverse range of food ingredients and additives is used, i.e. vegetable and animal proteins (Sousa *et al*., 2017, Campagnol*et al*., 2013; Lee *et al*., 2016) and polysaccharides: starches, gums, fiber, etc. (De Oliveira Faria*et al*., 2015; Han M. *et al*., 2017; Alves *et al*., 2016; Henning *et al*., 2016). It should be noted that in the manufacture of meat products, protein preparations are traditionally used to replace lean meat, rather than fat. This is due to the fact that replacing the fat with vegetable or animal proteins may lead to deterioration of consumer characteristics, in view of the fact that the resulting protein gels do not able to simulate fat functions in the product. For this purpose, carbohydrates are more successfully used, among which the inulin is of special interest. A large number of works in different countries are aimed at the development of low-calorie meat products with inulin, the use of which allows to significantly reduce the fat content in meat products (Furlan*et al*., 2014, Keenan *et al*., 2014, Shoaib *et al*., 2016, Silva-Vazquez*et al*., 2018). This natural polysaccharide in a hydrated form (inulin: water ratio 1: (1 to 2)) has properties that allow to simulate fat in the product resulting in delicate soft texture, white color, and absence of foreign odor and flavor. Preliminary studies have revealed the possibility of using inulin in a hydrated form to replace the fat in meat products in the amounts up to 50% of its content in the formulation, which helps to reduce fat content by more than 40% (Semenova*et al*., 2012). Being a dietary fiber, inulin has a beneficial effect on the function of gastrointestinal tract, significantly increases the absorption of minerals, decreases cholesterol level in blood, and markedly improves the metabolism of carbohydrates and lipids (Kumar *et al*., 2016; Han K.H. *et al*., 2017). However, taking into account that meat products containing inulin are multicomponent products, studies aimed at physiological evaluation of sausage with inulin as a fat substitute in comparison with traditional sausage are needed. In this connection, the aim of the work was to study the effect of cooked sausage with inulin on the clinical and physiological parameters of laboratory rats.

## 2. Materials and methods

### 2.1.Materials

#### *2.1.1. Samples*

For the experiment, cooked sausage was produced. The control sample of the cooked sausage contained 50 kg of lean pork, 28 kg of high-grade beef, 22 kg of fatback, water (25 l over the formulation), salting ingredients (sodium chloride, sodium nitrite), spices, food phosphates, and sodium ascorbate.

In the formulation of experimental products, 16 kg of fatback was replaced by 10 kg of inulin gel with inulin: water ratio of 1: 1.5, 2 kg of milk protein and 4 kg of hydrated egg protein. Cooked sausage was produced according to traditional technology (Kapovsky*et al*., 2017) with cooking at a core temperature of 72 ± 2 °C.

#### *2.1.2.Management and stunning of animals*

As a laboratory model, aged rats of the Wistar line at the age of 11 weekswith a body weight of 340-370 g were used. Animals were obtained from the Andreevka branch of the Scientific Center for Biomedical Technologies of the Russian Federal Medical and Biological Agency and passed quarantine for 14 days. Rats were randomly divideto 3 groups (n = 27): Group 1 was fed with the standard vivarium died (SVD) with addition of experimental cooked sausage; Group 2 consumed control samples

of cooked sausage; Group 3 was intact, and rats consumed SVD throughout the experiment. The standard diet of the vivarium consisted of cooked pearl barley and standard chow(Laboratorkorm, Russia). Ground samples of cooked sausage were introduced into the cooked barley. Diets of animals in all groups were balanced and contained equal amount of protein (10% of calories).

Animals were kept under similar conditions, i.e. temperature (20 ± 3 °C), humidity (48 ± 2%), illumination (light day 6.00 a.m. to 6.00 p.m.), in polysulfone cages (Tecniplast, Italy) with free access to water and feed. On every 4th day, animals were weighed on Ohaus electronic technical scales (AdventurerPro, USA), weight change was analyzed, and diet was calculated. Weighing and feeding of animals were performed daily at a strictly defined time (1.00 p.m. to 2.00 p.m.).

The experiment was conducted for 26 days. By the end of the experiment, the animals were stunned in the euthanasia chamber (VetTech, UK). From the right ventricle of the heart of stunned animals, clinical and biochemical analyses were carried out.

## 2.2.Methods

### *2.2.1.Methods for studying the physical and chemical properties of cooked sausage*

The mass fraction of protein in the sausage was determined as a result of mineralization of the Kjeldahl sample and a photometric measurement of color intensity of indophenol blue, which is proportional to the amount of ammonia in the mineralized sample. Fat content was determined by the method based on the extraction of total fat with hexane or petroleum ether with a boiling point of 50 to 60 °C in the Soxhlet extraction apparatus. The mass fraction of carbohydrates was determined by the calculation method, subtracting the values of moisture, fat, protein and ash mass fractions from the 100 g of the product.

Determination of the meat product color characteristics in the CIELab system was carried out using a spectrocolorimeter (Spectroton, Russia) while simultaneously measuring reflection coefficients of samples at 24 fixed wavelengths in increments of 13 nm in the visible spectral range from 380 to 720 nm, followed by mathematical processing of the measurement results by microprocessor controller integrated in the measuring unit.

To determine color stability during storage, the color stability criterion (U, %) was used (Semenova*et al*., 2007).

The shear force was determined using Instron-3342 universal testing machine, USA, with subsequent recording and export of measurement results to an Excel file.

The pH value was registered by a potentiometric method using Zamer-1 portable pH-meter (Russia).

The water activity was determined by the cryoscopy method using AWK-20 instrument (Germany).

### *2.2.2.Methods for studying the physiological parameters of laboratory rats*

#### *2.2.2.1.Clinical and biochemical blood analyses*

Clinical analysis of blood samples was performed on Abacus Junior Vet 2.7 automatic veterinary hematological analyzer (DiatronMesstechnik GmbH, Austria) using Diatron reagent kits. Blood serum studies were performed on BioChemSA biochemical analyzer (HTI, USA) using reagent kits (HTI, USA).

#### *2.2.2.2. Postmortem examination*

Postmortem examination was carried out by visual inspection of internal organs. The absolute weight of the liver, kidneys, spleen, and heart was determined by weighing on electronic scale with an accuracy of

± 0.001 g (AcculabVicon, USA), and the relative weight of organs (spleen, kidney, liver, heart) was calculated (Dzhimak*et al*., 2015).

### 2.3. Statistical analysis

Statistical processing of the data was carried out by the analysis of variance using Microsoft Excel 2010 and STATISTIKA software packages; the difference of the compared indicators was considered significant with a probability of difference more than 95% ($p < 0.05$).

## 3.Results and discussions

### 3.1. Physical and chemical properties of sausage

The replacement of fatback by inulin gel had no significant effect on pH, water activity and color indices (Table 1). The data obtained were consistent with the results of Furlan*et al.* (2014), who found that using of inulin makes it possible to simulate fat in minced semi-finished products while reducing fat content by 20-35% without changing the appearance and color of the minced meat and sensory properties of the finished product.

According to the results of studies, an increase of color stability in experimental sausage was found to be 4.1%, which is obviously explained by a decrease in ultraviolet-induced oxidation processes due to the introduction of inulin instead of fatback. The use of inulin contributed to an increase in shear force of experimental cooked sausage by 17.1% ($p < 0.05$) compared to control. The data obtained are consistent with the results of Derek *et al*. (2012), who found that with an increase in the proportion of fat replacement with inulin, the density of sausage increases, which may be due to the formation of interacting microcrystal groups forming a gel network.Studies of the chemical composition showed that the introduction of inulin reduced the fat content by 47.3% ($p < 0.05$), while the caloric content decreased by 28.8% ($p < 0.05$) compared to the control (Table 2).

**Table 1.**The results of physical and chemical studies of cooked sausage

| Parameters | Sample | |
|---|---|---|
| | **Control** | **Experimental** |
| **pH** | 6.24±0.04 | $6.28±0.03^{ns}$ |
| **Shear force, Pa** | 21.7±1.2 | $25.4±0.5^{s}$ |
| **Water activity, units** | 0.9742±0.006 | $0.9744±0.0003^{ns}$ |
| **Lightness, units** | 69.7±2.3 | $67.6±3.1^{ns}$ |
| **Redness, units** | 9.4±0.6 | $9.7±0.2^{ns}$ |
| **Yellowness, units** | 14.0±0.7 | $14.6±0.3^{ns}$ |
| **Color stability, %** | 71.3 | $75.4^{ns}$ |

$^{ns}$not significant $p \geq 0.05$; $^{s}$ significant at $p < 0.05$.

**Table 2.**Chemical composition of cooked sausage

| Sample | Mass fraction in the product, % | | | Caloric content, kcal |
|---|---|---|---|---|
| | **proteins** | **fat** | **carbohydrates** | |
| Control | 12.3±0.5 | 20.5±2.1 | - | 233.7 |
| Experimental | $13.7±0.8^{ns}$ | $10.8±0.9^{s}$ | $3.6±0.4^{s}$ | $166.4^{s}$ |

$^{ns}$not significant $p \geq 0.05$; $^{s}$ significant at $p < 0.05$.

### 3.2.Clinical and physiological parameters of laboratory rats

#### *3.2.1.Changes in weight of laboratory rats*

Observations of rats in Groups 1 and 2, in which the experimental sausage was added to the diet, as well as for the intact.

Group 3 animals consuming the standard diet, did not reveal any abnormalities in the physiological state. The sausages in the diet was completely eaten. Weight dynamics of the animals showed that the rats in Groups 1 and 2 gained weight more rapidly in the first day of the experiment (Figure 1). Group 1 animals intensively gained weight on the first 4 days (on average, up to 7 g/day), and in the period from 4th to 26th day, weight stabilization was noted, when the rats gained on average not more than 4 g/day. Group 2 rats also gained weight quite intensively during the first 4 days. Further observation showed an increase in animal weight on average of 5 g/day. Intact animals in Group 3 consistently gained weight throughout the experiment on average of 4-6 g/day. Weight gain by the end of the experiment was as follows: animals in Group 1 consuming experimental products - 13.1 ± 0.6%, rats in Group 2 consuming control samples – 15.0 ± 2.8%, intact animals in Group 3 – 15.4 ± 2.3%.

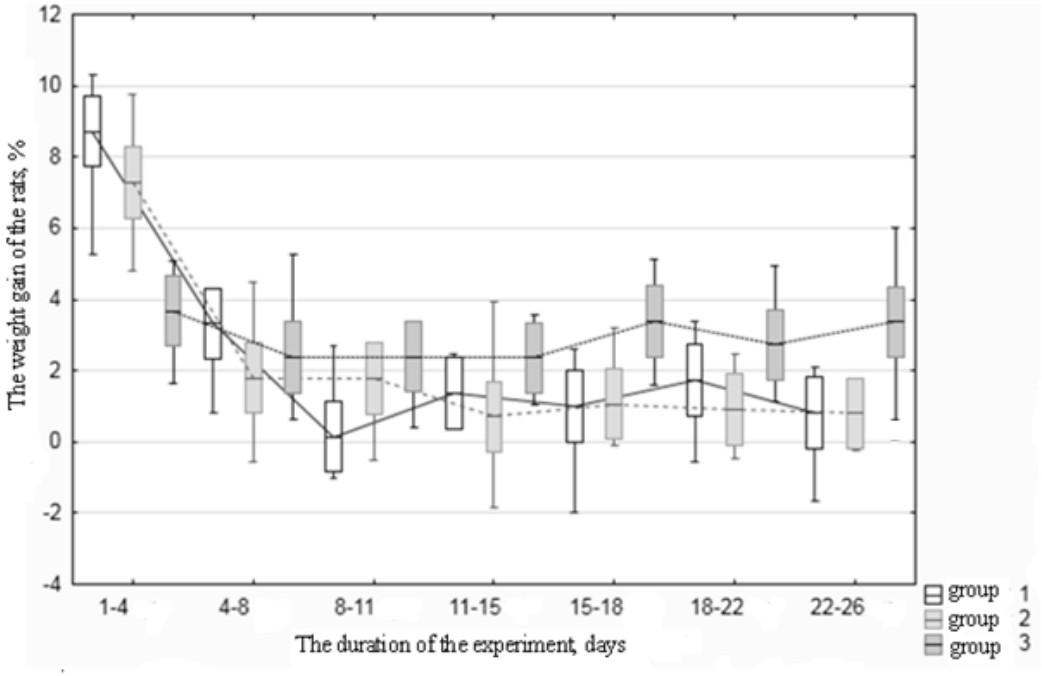


**Figure 1.**Body weightdynamics of experimental rats during the experiment

#### *3.2.2. Clinical blood analysis*

The results of clinical blood analysis showed some changes in the hematological indices of experimental rats compared to intact animals.

Redistribution of white blood cells (WBC) was most pronounced in animals fed with experimental and control samples of cooked sausages, which was characterized by a significant decrease in lymphocyte content and an increase in mixture of monocyte, eosinophil, basophil and immature cells by up to 40% (Figure 2). Group 1 rats also showed an increase in granulocytes by 35%.

With respect to hematological parameters characterizing the functional activity of red blood cells of Group 1 rats, a statistically significant decrease in the volume of erythrocytes compensated by an increase in hemoglobin was revealed with a red blood cell content (erythrocytes and hematocrit) not significantly different from intact rat values (Table 3). Other parameters characterizing the functional activity of platelets of the rats in Groups 1 and 2 were not significantly different from intact animals.

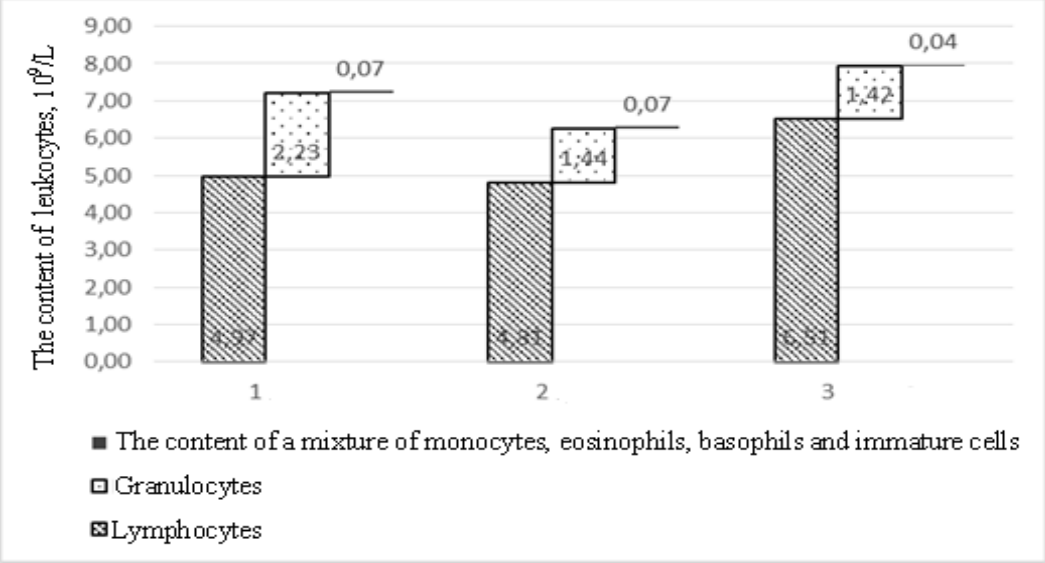


**Figure 2.**Differential blood count of experimental rats

**Table 3.**Morphological indicators of animal blood characterizing the functional state of erythrocytes

| Parameters | Group of animals | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| **RBC, $10^{12}$/L** | 8.19±0.18$^{a}$ | 7.98±0.24$^{a}$ | 8.12±0.10$^{a}$ |
| **Hemoglobin, g/L** | 147.22±2.23$^{ab}$ | 144.05±3.74$^{b}$ | 150.75±1.26$^{a}$ |
| **Hematocrit, %** | 41.25±0.80$^{a}$ | 42.64±1.14$^{a}$ | 44.31±0.56$^{a}$ |
| **Mean corpuscular volume, µm$^3$** | 49.44±1.32$^{b}$ | 53.67±1.21$^{a}$ | 54.51±0.58$^{a}$ |
| **Mean corpuscular hemoglobin concentration, g/L** | 353.13±3.14$^{a}$ | 334.01±4.20$^{b}$ | 337.80±2.05$^{b}$ |
| **Red cell distribution width, %** | 16.34±0.43$^{a}$ | 15.84±0.32$^{a}$ | 15.44±0.34$^{a}$ |

Means in the same column with different superscript letters are significantly different ($p<0.05$).

### *3.2.3. Biochemical blood analysis*

The results obtained in the biochemical analysis of serum from experimental animals are presented in Table 4. Evaluation of protein metabolism indices revealed non-significant increase in total protein content in Group 1 animals consuming experimental products, mainly due to the albumin fraction, with a statistically significant increase of urea level. In Group 2 rats consuming control samples, a slight but significant decrease in total protein content was observed by up to 10%, as compared to the intact Group 3. An increase in glucose

level by more than 20% in serum of 33% of experimental rats in Group 1 was observed, but the averaged index was not statistically different from the values of comparison groups. With regard to bilirubin, one of the key indicators of pigment metabolism, a significant increase in the concentration of the total fraction of this compound in the blood of the rats in Groups 1 and 2 was observed by 38.3% and 27.2%, but these values did not exceed the physiological normal rates for rats of this age.

Compared to intact rats in Group 3, study of a number of serum enzymes in experimental rats revealed in Group 1 a significant increase in the activity of cytoplasmic enzyme ALT (by up to 40%), with a slight non-significant change in the activity of AST (by more than 10%), which has mitochondrial cytoplasmic localization. In group 1, the De Ritis coefficient reflecting the ratio of AST/ALT activities was 3.28 ± 0.77, which is more than 1.5 times lower than the value of this coefficient in the group of intact rats.

The noted tendency to increase the total protein content, including albumin, glucose, and total bilirubin with the increase in the activity of intracellular enzymes of aminotransferase group may indicate an enhancement in the functional activity of the liver and, as a result, acceleration of protein and carbohydrate catabolism in animals, in which diet experimental meat products were added.

When analyzing the biochemical parameters characterizing the lipid metabolism of experimental animals, a statistically significant decrease in the total cholesterol content (Figure 3) and triglycerides (Figure 4) by up to 25% was found, with non-significant decrease in high-density lipoproteins (HDL) (Figure 5) and low density lipoproteins (LDL) (Figure 6) (by not more than 10%) compared to the values of intact animals. In Group 1 consuming experimental sausage, there was a slight decrease in cholesterol, triglycerides and LDL level ($p > 0.05$). It should be noted that a significant increase in lipase content in the serum of animals in Group 1 detectable at 68.72 ± 3.42 U/L was observed versus 36.27 ± 3.04 U/L and 55.13 ± 5.74 U/L in control rats of Group 2 and intact rats of Group 3, respectively. The data obtained are consistent with the results of Kumar *et al.* (2016), who established that when consuming soluble fiber, there is an increase in the metabolism of carbohydrates and lipids.

**Table 4.**General biochemical indicators of animal blood

| **Parameters** | **Normal** | **Group of animals** | | |
|---|---|---|---|---|
| | | **1** | **2** | **3** |
| **Total protein, g/L** | 50-80 | 76.40±1.46$^{a}$ | 70.96±0.70$^{b}$ | 75.76±2.07$^{a}$ |
| **Albumin, g/L** | 30-50 | 42.22±1.64$^{a}$ | 39.58±1.06$^{a}$ | 42.69±1.72$^{a}$ |
| **Creatinine, µmol/L** | 9-70 | 57.84±1.17$^{a}$ | 58.34±1.71$^{a}$ | 59.25±0.64$^{a}$ |
| **Urea, mmol/L** | 4.3-8.6 | 7.20±0.59$^{a}$ | 5.47±1.06$^{b}$ | 5.42±0.63$^{b}$ |
| **Glucose, mmol/L** | 7.7-12.2 | 11.42±2.98$^{a}$ | 8.87±1.72$^{a}$ | 9.02±2.35$^{a}$ |
| **Bilirubin (total), µmol/L** | 0-8.5 | 3.13±0.25$^{a}$ | 2.65±0.53$^{ab}$ | 1.93±0.49$^{b}$ |
| **Aspartate aminotransferase (AST), U/L** | 20-180 | 158.42±14.65$^{a}$ | 151.12±11.75$^{a}$ | 178.71±26.79$^{a}$ |
| **Alanine aminotransferase(ALT), U/L** | 10-80 | 46.73±6.75$^{a}$ | 27.63±8.63$^{b}$ | 28.68±6.43$^{b}$ |
| **Activity Ratio (AST/ALT)** | - | 3.28±0.77$^{a}$ | 5.04±0.66$^{ab}$ | 5.36±0.89$^{b}$ |

Means in the same column with different superscript letters are significantly different ($p<0.05$).

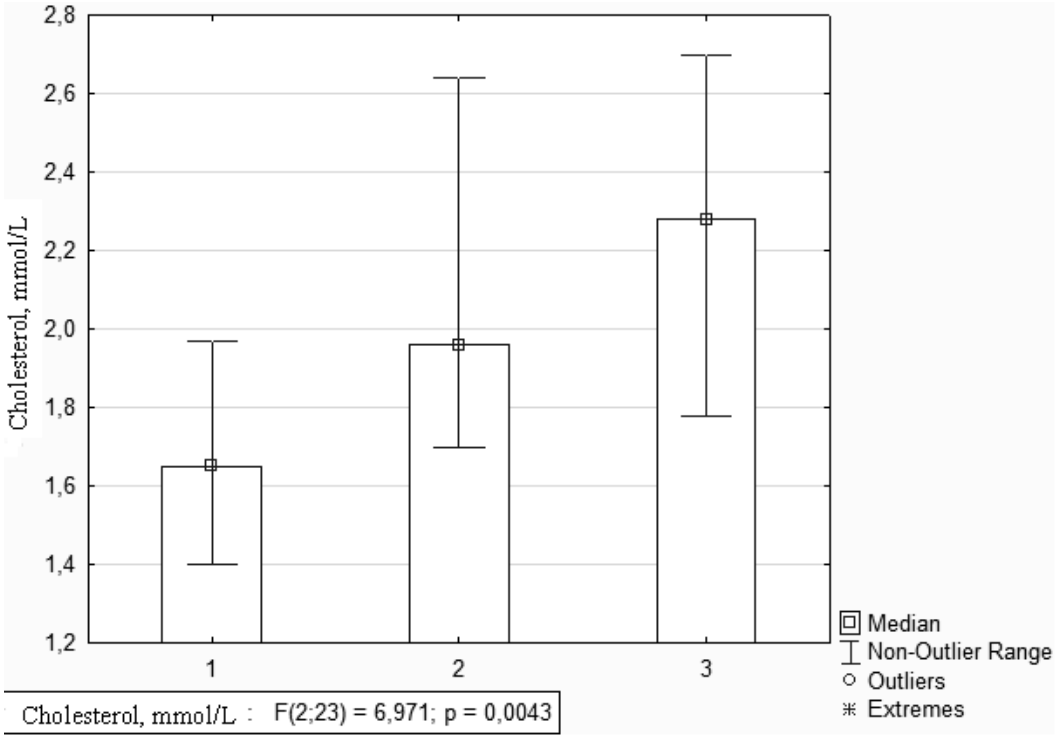


**Figure 3.** Cholesterol content in the animal blood

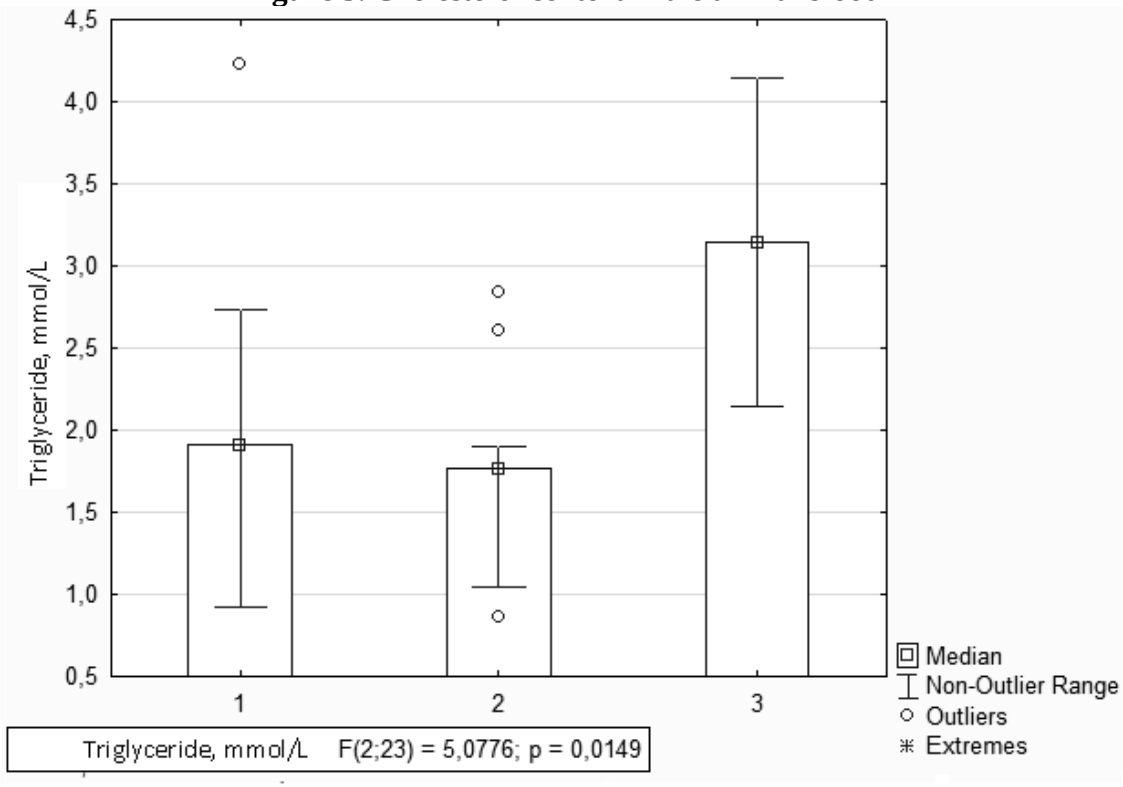


**Figure 4.** Triglyceride content in the animal blood

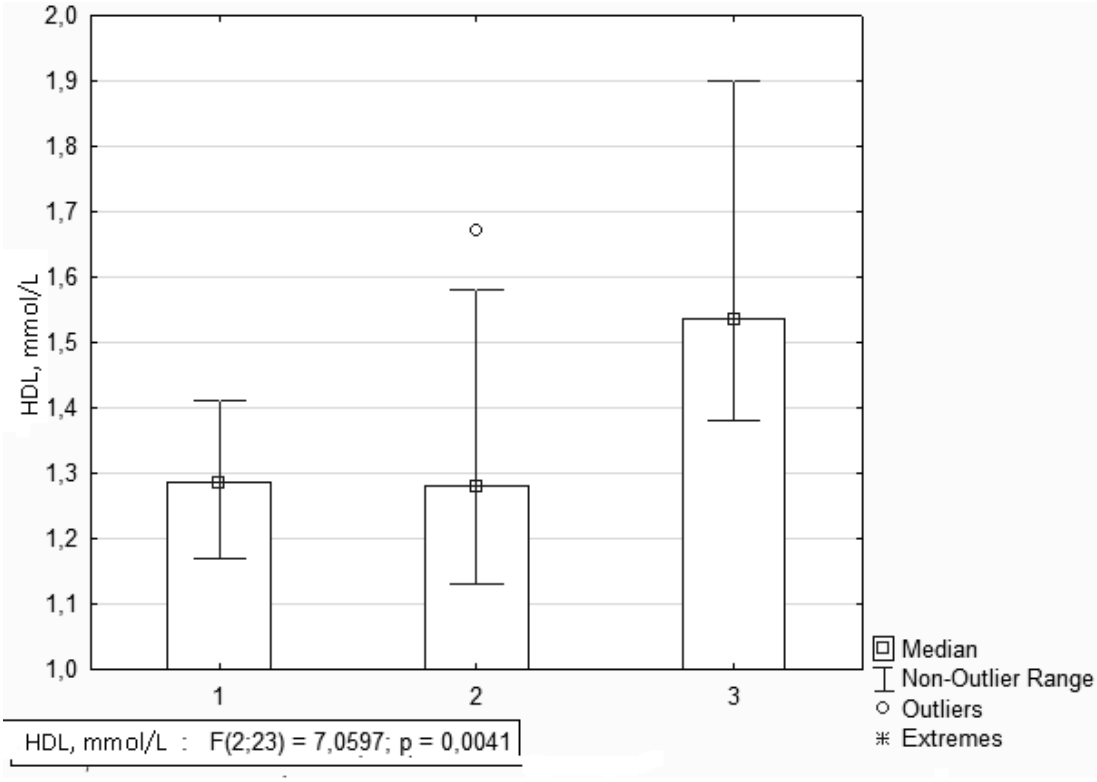


**Figure 5.**HDL content in the animal blood

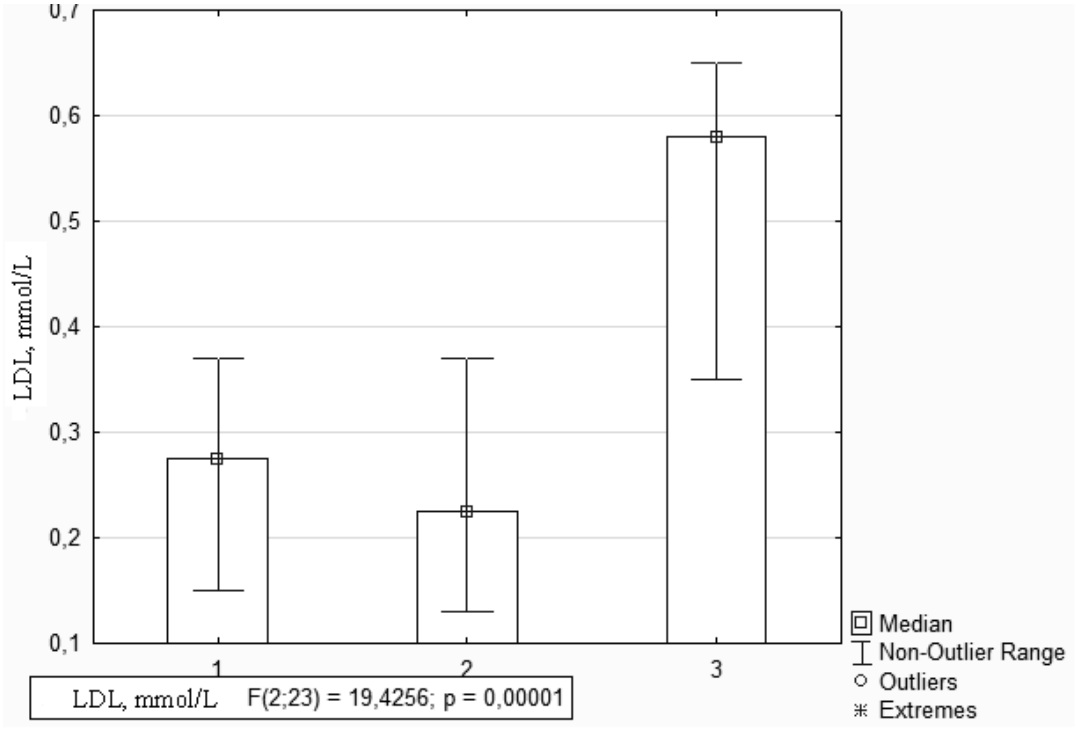


**Figure 6.**LDL content in the animal blood

### *3.2.4. Postmortem examination*

Postmortem examination of animals did not reveal any significant abnormalities in the state of the internal organs (digestive tract, respiratory system, circulatory and hematopoiesis organs, urinary system, and internal secretion organs); there were no significant changes in the relative weight of the organs analyzed.

## 4. Conclusions

Thus, during this work, it has been established that the use of inulin gel instead of fatback allowedto produce cooked sausage with a reduced fat content without impairing sensory properties.

As a result of the biological experiment, it can be stated that 26-day introduction of low-calorie meat products with inulin into the diet of aged laboratory rats promotes a decrease in the average weight gain of animals from 7 g to 4 g per day, while the body weight of animals at the end of the experiment exceeded the initial mass of experimental rats by 13%, while in control and in intact groups this indicator was 15%.

Changes in hematological and biochemical blood indices of experimental animals were identified that were characterized by redistribution of white blood cells: a significant decrease in lymphocyte count by more than 20% with an increase in monocyte, eosinophil, basophil, immature cell and granulocyte count by up to 40%. The increase in total protein, albumin, glucose, total bilirubin content and aminotransferase activity indicates an enhancement in the functional activity of the liver and, as a result, acceleration of protein and carbohydrate catabolism, as well as intensification of active proliferation of lymphoid tissue cells.

The study of key lipid metabolism indices, i.e. total cholesterol, triglycerides, high-density lipoproteins (HDL), and low density lipoproteins (LDL), revealed a significant decrease in these parameters in rats consuming cooked sausage with inulin: total cholesterol and triglycerides by up to 25%, HDL and LDL by 10 %. Lipase increase in serum of animals in the experimental group by more than 20% was also revealed.

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# ESTIMATING THE SHELF LIFE OF A MAYONNAISE MADE FROM SACHA INCHI (*PLUKENETIA VOLUBILIS* L.) OIL AND DUCK (*ANAS PLATYRHYNCHOS* L.) EGG YOLK

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**ABSTRACT**

Mayonnaise is one of the most industrially produced and consumed food emulsions. Consistent mostly of oil, it is susceptible to deterioration by peroxidation of lipids that is manifested in sensory unpleasant characteristics associated with the chemical species that are produced in this process, which reduce their shelf life. This work studied the shelf life of a mayonnaise made from sacha inchi (*Plukenetia volubilis* L.) oil and duck (*Anas platyrhynchos* L.) egg yolks, using the peroxide value as an indicator. Three storage temperatures (12, 22 and 32 °C) were used to obtain the specific reaction constants (0.112, 0.142 and 0.359 mEq-peroxide·day$^{-1}$, respectively) to determine the activation energy (41752 J·mol$^{-1}$) and shelf life for each temperature. The evolution of peroxide value was similar to other mayonnaise formulations.

## 1. Introduction

Mayonnaise is one of the oil-based products most consumed and produced by the food industry worldwide. Its oily nature makes it susceptible to deterioration by oxidation which is manifested by different compounds of a disagreeable odor. The most commonly used approach to delay rancidity reactions and prolong the shelf life of this product has been the use of synthetic or natural antioxidant compounds (Ghorbani Gorji *et al*., 2016) with great success in most cases except for a deterioration in sensory characteristics when using antioxidants from natural extracts.

Since interest in mayonnaises obtained from oils rich in unsaturated fatty acids has increased (Jacobsen *et al*., 2001; Di Mattia *et al*., 2015), it is necessary to study the effect of different parameters that may affect the product during storage. While light is mostly controlled by packaging, the temperature is usually the ambient temperature in most cases. When mayonnaises formulated with a different lipid profile were investigated, the best analytical tests for early oxidation were the peroxide value and the thiobarbituric acid test (Hsieh and Regenstein, 1992). had also determined that peroxide value is useful in predicting the potential shelf life of mayonnaise. Wills and Cheong (1979) had also determined that peroxide value is useful in predicting the potential shelf life of mayonnaise.

Sacha inchi (*Plukenetia volúbilis* L.) oil has a higher content of polyunsaturated fatty acids than olive and fish oil, and a lower content of saturated fatty acids (Paucar-Menacho *et al*., 2015). Obtained from the cold pressing of the seeds, this oil has an initial peroxide value between 5.2 and 5.6 mEq-peroxide·kg$^{-1}$ oil (Castaño *et al*., 2012).

The aim of this study was to estimate the shelf life of a mayonnaise obtained from sacha

inchi oil using duck (*Anas platyrhynchos* L.) egg yolk as an emulsifier. The peroxide value was the variable used to predict shelf life by studying the behavior of its evolution in samples stored at different temperatures.

## 2. Materials and methods

### 2.1. Materials

Sacha inchi oil (*Plukenetia volúbilis* L.) was purchased from Empresa Agroindustrias Amazónicas (Lima, Peru) in bottles of 250 ml each. The eggs of duck (*Anas platyrhynchos* L.) were acquired from agricultural breeding areas in the city of Arequipa, Peru. The raw materials were stored refrigerated at 5 °C until use.

### 2.2. Preparation of the emulsion

The duck egg yolk was pasteurized at 56°C for 5 minutes and mixed with sacha inchi oil at 10500 RPM for 1 minute in a variable speed blender. Emulsification was performed on the same equipment at 12000 RPM for 3 minutes. No antioxidants were used in the formulation.

### 2.3. Packaging and storage

The mayonnaise samples were packed in 150 x 100 mm aluminum/polyethylene (alupol) bags and 500 grams capacity each, sealed using an electric sealer. The product was stored at three different temperatures for 31 days: 12°C (285.15 K), 22°C (295.15 K) and 32°C (305.15 K), using a laboratory heating oven.

### 2.4. Determination of peroxide value

Oil extraction from mayonnaise samples was carried out according to the procedure 983.23 described in AOAC (2006). A sample of 15 g was weighed in a 500 mL flask containing 80 mL methanol and 40 mL chloroform. The contents were stirred in a water bath between 45°C and 50°C for 15 min. Then 40 ml of chloroform was added and mixed for 5 minutes. A volume of water of 40 mL was then added and mixed for 1 minute. It was left to stand until the phases were separated and the oily phase was extracted using a pipette to centrifugate the extract at 10500 RPM. The chloroform of the oily extract was evaporated in a water bath.

The method described in ISO (2017) with modifications for determination of the peroxide value was used. A solution of 50 mL of acetic acid: chloroform (3:2 ratio) was added to the extracted and weighed oil sample and the mixture was agitated. Then 0.5 mL of a saturated potassium iodide solution was added, allowed to react for exactly 1 minute and 0.5 mL of starch solution was added. Immediately afterwards 30 mL of distilled water was added. Titration was performed with 0.06 M sodium thiosulfate solution gradually and steadily until the bluish color disappeared. Previously the same procedure was performed using a blank, obtaining a value that was subtracted from the experimental results.

The units of measurement of the peroxide value used were the milliequivalents peroxide per kilogram of oily sample (mEq-peroxide·kg$^{-1}$). Equation 1 was used to calculate the peroxide value.

$$PV = \frac{1000(V-V_b)\cdot c}{m} \quad (1)$$

Where PV is the peroxide value (mEq-peroxide·kg$^{-1}$), V is the volume of sodium thiosulphate solution used for the determination (mL), Vb is the volume of sodium thiosulphate solution used for the blank, c is the concentration of sodium thiosulphate solution and m is the mass (g) of the sample.

### 2.5. Packaging and storage

Food deterioration has been found to follow zero-order or first-order kinetic models. In foods with a high fat or lipid content, peroxidation reactions predominate, following a behaviour of zero order (Labuza, 1984). The model for the reaction of zero order is presented in Equation 2, according to García Baldizón and Molina Córdoba (2008).

$$-\frac{dX}{dt} = k \quad (2)$$

Where X is the concentration of the chemical species of interest, k is the specific reaction constant and t is the time. For this work

X was the peroxid value and t is expressed in days.

Since k is time-dependent, the Arrhenius model was used to describe this relationship, according to Equation 3.

$$k = A^{(E_a/RT)} \quad (3)$$

Equation 3 is then cleared to obtain a linear form in Equation 4, where A is the frequency factor, $E_a$ is the activation energy, R is the constant of the ideal gases (8.3143 $J \cdot K^{-1} \cdot mol^{-1}$) and T is the absolute temperature in Kelvin. In this way, the slope of the line allows the activation energy to be determined.

$$\ln(k) = \ln(A) - \frac{E_a}{R} \cdot \frac{1}{T} \quad (4)$$

### 2.6. Statistical analysis

Simple linear regression analysis and graphs were obtained using the R programming language and environment for statistical computation (R Development Core Team, 2008).

## 3. Results and discussions

### 3.1. Evolution of peroxide value

The results of the peroxide value during storage at different temperatures are shown in Table 1. Peroxide values increased over time for all storage temperatures, although at 285.15 K the values seem to stabilize after 17 days so the value of r was relatively low (Table 2) but significant (p-value < 0.05). The highest linearity between peroxide value and storage time was observed at 305.15 K ($r^2$ = 0.9756). Figures 1, 2 and 3 show the peroxide values for each storage temperature. The plots showed that confidence intervals were narrower as the temperature increased.

The role of stabilizers and antioxidants in decreasing the appearance of oxidation products during mayonnaise storage has been confirmed as very important in other studies. The emulsions obtained with a high percentage of oil rich in polyunsaturated fatty acids and stabilizers with chelating and radical scavenging properties have had very low levels of PV, less than 0.93 mEq peroxides·$kg^{-1}$ oil at 4 weeks of storage (Yesiltas *et al*., 2017). As no antioxidants or stabilizers have been used in this study, peroxide values have been higher.

**Table 1.** Peroxide values during storage of mayonnaise at different temperatures

| Day | Peroxide value (mEq-peroxide·$kg^{-1}$) | | |
|---|---|---|---|
| | 285.15 K | 295.15 K | 305.15 K |
| 0 | 2.91 | 2.91 | 2.91 |
| 7 | 4.46 | 5.08 | 6.46 |
| 17 | 6.22 | 6.91 | 8.18 |
| 24 | 6.28 | 7.03 | 12.18 |
| 31 | 6.36 | 7.50 | 14.36 |

**Table 2.** Correlation matrix (Pearson's correlation coefficients) of peroxide values (PV) and storage time (days)

| Variable | Days | PV at 285.15 K | PV at 295.15 K | PV at 305.15 K |
|---|---|---|---|---|
| Days | 1.000 | 0.920 | 0.937 | 0.988 |
| PV at 285.15 K | | 1.000 | 0.995 | 0.887 |
| PV at 295.15 K | | | 1.000 | 0.913 |
| PV at 305.15 K | | | | 1.000 |

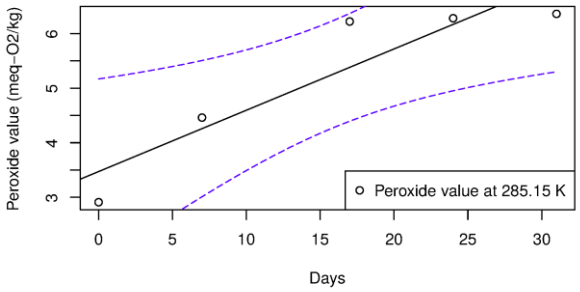


**Figure 1.** Evolution of mayonnaise peroxide values at 285.15 K (12 °C). Purple lines represent the confidence intervals at 95%.

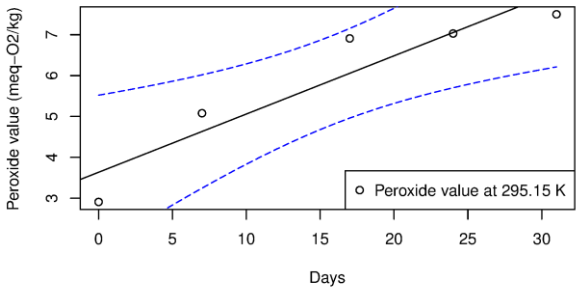


**Figure 2.** Evolution of mayonnaise peroxide values at 295.15 K (22 °C). Purple lines represent the confidence intervals at 95%.

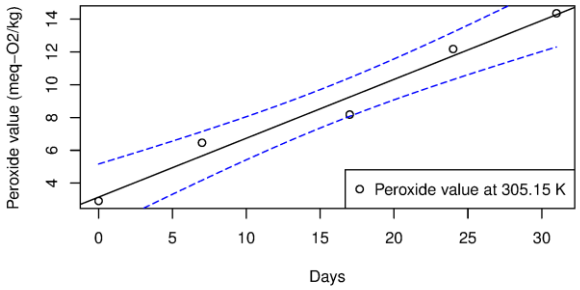


**Figure 3.** Evolution of mayonnaise peroxide values at 305.15 K (32 °C). Purple lines represent the confidence intervals at 95%.

A mayonnaise-based dressing (the unsaturated fatty acid content was 83.74%), to which a hydrolyzed extract of rapeseed cake was added, had peroxide values of 4.16 to 4.42 mEq-peroxide·kg$^{-1}$ oil which, after 6 weeks of storage at 4 °C, increased to the range of 13.48 to 15.19 mEq-peroxide·kg$^{-1}$ oil, and then decreased by the generation of secondary oxidation products (Kim and Lee, 2017). The authors observed that the peroxide values were significantly lower than those of the control without adding the extract. Our peroxide value results at the same storage temperature were lower at the beginning (Table 1) and would probably have been lower than those reported after 6 weeks considering the projected value. More similar to our initial peroxide values were those reported for mayonnaise mixed with mango using egg yolk and mustard as emulsifiers, which amounted to less than 3.183 mEq-peroxide·kg$^{-1}$ oil (Sethi *et al*., 2017).

After 35 days of storage at 20 °C, a mayonnaise made from soybean oil (70%) reached a peroxide value of 13.06 mEq-peroide·kg$^{-1}$ oil, from an initial value of 6.6 mEq-peroxide·kg$^{-1}$ oil. At 56 days the value reached 36.8 mEq-peroxide·kg$^{-1}$ oil. The study authors (Phuah *et al*., 2016) noted that this large increase in peroxide value may have been due to the high percentage of unsaturated fatty acids in soybean oil, although our results at a similar temperature (22°C) with oil also rich in unsaturated fatty acids were lower.

After 5 weeks of storage at 25 °C, another mayonnaise made from soybean oil (70%) had a peroxide value of 9.65 mEq-peroxide·kg$^{-1}$ oil. The mayonnaise samples from the same study (Azhagu Saravana Babu *et al*., 2016) to which a seed extract of *Cucumis sativus* was added had significantly lower peroxide values. Our results at a similar temperature (22 °C) allowed us to predict that at 35 days (5 weeks) the peroxide value would be 8.62 mEq-peroxide·kg$^{-1}$ oil, which is very close to the reported value.

During storage at 37 °C, mayonnaise samples (78% oil) reached a peroxide value of 7.88 mEq-peroxide·kg$^{-1}$ oil in the third week, and 7.84 mEq-peroxide·kg$^{-1}$ oil in the sixth week (Kwon *et al*., 2015). The trend of our results at a lower temperature (32 °C, Figure 3) indicates that our samples showed greater oxidation in comparison, although the temperature was lower by 5 °C. At higher storage temperatures (45 °C), it has been reported (Bholah *et al*., 2015) that samples of

mayonnaise from sunflower oil without the addition of an antioxidant extract had a peroxide value of more than 6 mEq-peroxide·kg$^{-1}$ oil, reaching approximately 50 mEq-peroxide·kg$^{-1}$ oil after 15 days. Therefore, it is confirmed that temperature plays a very important role in the spread of auto-oxidation.

### 3.2. Specific reaction constants and activation energy

The specific reaction constant (k) at 285.15 K was 0.112 mEq-peroxide·kg$^{-1}$, 0.142 mEq-peroxide·kg$^{-1}$ at 295.15 K and 0.359 mEq-peroxide·kg$^{-1}$ at 305.15 K. It was observed that the k value increases with temperature as expected, remarkably above 22 °C. From the Figure 4 the equation of the regression line (r2 = 0.8832) was ln(k) = 15.307 - 5021.653 · (1/T). Using the slope of this regression line and Equation 4 the activation energy was determined as 41752 J·mol$^{-1}$.

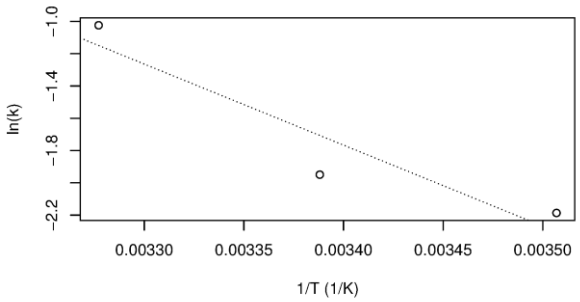


**Figure 4.** Logarithm of the specific constant of reaction in relation to the inverse of temperature.

It has been suggested that phospholipid peroxidation of mayonnaise is predominantly initiated by radical oxidation (via triplet oxygen) rather than singlet oxygen oxidation throughout the manufacturing and storage process (Kato *et al*., 2017). Unsaturated fatty acids, such as those present in sacha inchi oil, are more susceptible to oxidation due to the low activation energy in the initiation of free radical formation for triplet oxygen auto-oxidation (Min and Boff, 2002). The low activation energy obtained from our results would confirm that peroxidation of the mayonnaise samples was predominantly due to self-oxidation, as the temperature has had an appreciable effect (when greater than 22 °C) and the samples were protected from light by the aluminium/polyethylene container. Temperature has been reported (Yang and Min, 1994) to have little effect on the reaction rate of singlet oxygen oxidation in spite of the low activation energy required (0 - 25120.8 J·mol$^{-1}$).

### 3.3. Shelf life

Using the k values and the intercepts from the regression equations obtained to fit the peroxide values for Figures 1, 2 and 3, there was possible to estimate the shelf life considering the limit of 7.96 mEq-peroxide·kg$^{-1}$ oil established sensorially by García Baldizón and Molina Córdoba (2008) for mayonnaise. The relationship was near lineal (Figure 5, $r^2$ = 0.9685) and the shelf life for the three storage temperatures (12, 22 and 32 °C) as estimated as 40, 30.4 and 12 days respectively, using the same regression equations from Figures 1, 2 and 3.

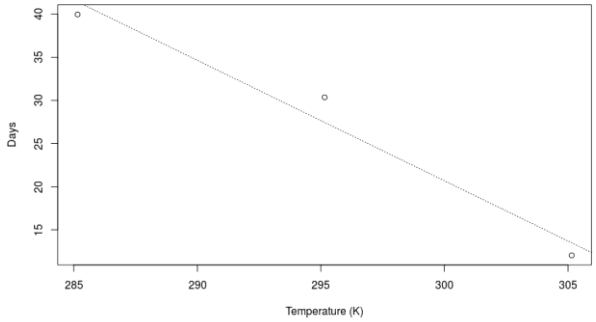


**Figure 5.** Estimated shelf life for mayonnaise stored at different temperatures.

### 4. Conclusions

This work estimated the shelf life of a mayonnaise made from sacha inchi oil and duck eggs, using the peroxide value as an indicator. A notable effect of the storage temperature on the peroxide value of the samples was observed during the storage time (31 days). Specific reaction constants (k) increased with temperature and activation energy would allow auto-oxidation in the absence of light due to

packaging. The estimated shelf life for storage temperatures of 12, 22 and 32 °C was 40, 30.4 and 12 days respectively.

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# MODELLING OF OSMOTIC DEHYDRATION PROCESS OF PEAR (*PYRUS COMMUNIS* L.) IN TERNARY SOLUTIONS OF SUGAR AND CALCIUM SALT USING RESPONSE SURFACE METHODOLOGY

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**ABSTRACT**

The holistic effects of temperature (40-60°C), sugar concentration (40-60°B), calcium chloride (1-3%) and immersion time (120-180minutes) were studied during the Osmotic Dehydration (OD) of Pear Sp. in ternary solutions. The Box Behnken Design of Response Surface Methodology (R.S.M.) was used to optimize various process variables like Water Loss, Solids Gain, Weight Reduction and Overall Acceptability by sensory evaluation of the Osmosed Product. Thereby establishing these as the Response Variables during Osmotic Dehydration of Pear. Optimized conditions were found to be 59.57°C temperature, 53.73°B sugar concentration, 2.27% calcium lactate salt and 151.9 minutes immersion time; for minimizing solid gain and maximizing water loss, weight reduction and overall acceptability.

## 1. Introduction

Pear (Pyrus communis L.) originally belongs to temperate climate zone of Europe originated in Asiatic region (Sharma et al. 2003, Park et al. 2002). The pear fruit contains natural source of Vitamin C, B Complex Vitamins (folates and riboflavin), Vitamin K, organic and fatty acids, volatiles and minerals such as copper, iron, potassium, magnesium and fiber (Pavelkic et al., 2015, Amiripour et al., 2015). It is mostly liked by the consumers due to its low calorific value and high nutritive value (Salim et al., 2016) while volatile compounds present in pear contributes to its flavour. The colour of the pear depends on its pigments mainly chlorophyll (green) and carotenoid (yellow) (Pattee, 1985, Park et al., 2002). The chemical composition of pears influences its acceptable organoleptic characteristics (Pavelkic et al., 2015). According to FAO, 2014 data, pear is cultivated in 1574446 ha of area in the world while 42280 ha of area in India. World production of pear in 2014 was about 25798644 tonnes while that in India was 316700 tonnes (FAO, 2014). Mostly fresh pear is processed to make juices, alcoholic beverages, compotes, sweet courses, fruit yoghurt, marmalades and jams (Pavkov et al., 2010) while dried pears are used in bakery products and gravies (Pavelkic et al., 2015).The traditional preservation method is drying of matured pear fruit, which involves removal of moisture content of the fruit by hot air drying, utilizing various techniques. However, due to degradation in the nutritional quality of dried products, this process is not so effective. Removal of moisture from the fruit can also be done by method of osmotic dehydration. Osmotic dehydration (OD) is a technique which is based on the immersion of fruits in a hypertonic solution which creates a high osmotic pressure on the dipped fruits and vegetables and

lowering its water activity (Raoult-Wack, 1994, Salim, 2016). However, the solute gain is less in comparision to the water loss from the fresh fruits and vegetables due to the semi-permeability of the cell membranes (Ahmed et al., 2016). OD depends on the properties of the biological material and complexity of each material varies from tissue to tissue and hence it is very challenging to optimize the processes and design the equipments for processing the biological materials (Tappi et al., 2017, Fernandez et al. 2004).

Ready to use intermediate moisture (IM) food products produced by Osmotic Dehydration (OD) are gaining importance now a days owning to its high nutritive content in comparison to be produced by any other drying methods (Pavelkic et al., 2015). This is due to the reason that OD has little effect on the flavour of the final product and its nutrients is preserved during the process. OD also provides benefits in decreasing the energy cost and retains the colour of the fruits and vegetables by inhibiting the browning of enzymes present in the food product (Salim et al., 2016, Pavelkic et al., 2015). OD modifies food composition in addition to water removal from the plant tissues. So, if the product is impregnated with desirable solutes then, it can improve the nutritional and sensorial characteristics of the final product (Tappi et al., 2017; Akbarian et al., 2014; Silva et al. 2014; Barrera et al., 2004). Selection of solute as an osmotic agent in the osmotic solution is a fundamental issue as it alongwith affects the dehydration kinetics and process cost, it also has an effect on the organoleptic and nutritional properties of the osmosed product. Several authors have considered Sucrose (Suc) as an optimal osmotic agent as it has higher efficiency than glucose (Tappi et al., 2017, Saputra, 2001) whereby reducing the enzymatic browning and aroma losses (Tappi et al., 2017; Cortellino et al., 2011; Qi et al., 1998; Lenart, 1996). Various investigators have performed OD with calcium salt in osmotic solutionmwhich has been used to increase the firmness of plant tissue while at the same time increasing the process efficiency, restricting sugar gain as sugar has higher molecular weight than the calcium salt it remains at the surface of the plant tissue and selectively allowing calcium to enter the tissue and here by at the same time increasing the water loss (Tappi et al., 2017; Mavroudis et al., 2012; Ferrari et al., 2010; Pereira et al., 2006). Pereira et al., (2006) stated that calcium salt disintegrates to form pectic polymers which by cross linking reinforces cell walls of the plant tissue and hence is able to reduce the damage from dehydration. When the conc. increases, damage to cell membrane may occur as reported by Anino et al., (2006). Also calcium being the desired solute, its salt have been used in osmotic solutions as a method for getting a fortified product high in nutrition, hence increasing the consumer intake due to being a calcium fortified product (Tappi et al., 2017; Silva et al. 2014; Barrera et al., 2004). It has also been stated that due to the addition of calcium salt the metabolic activity and respiration rates of different fruit species gets decreased (Tappi et al., 2017; Castello et al., 2010, Luna-Guzman et al., 1999; Lester, 1996) and therefore the stability of the product during storage gets potentially enhanced, especially considering that a lower respiration rate may lead to a longer shelf life. In addition, calcium present in the fruit affects the membrane and cell wall structure and functioning (Tappi et al., 2017; Maurel, 2007; Peiter et al., 2005).

Response surface methodology (RSM) is a statistical technique used to optimize the process variables in an experiment design and model development of a process. It has been widely used as an effective method for process and product improvement (Maran et al., 2013a, Maran et al., 2013b, Maran et al., 2013c). It helps in mapping a response surface of different variables to optimize the responses or for the selection of operating conditions or consumer requirements (Maran et al., 2013 c). It reduces the number of experiments, helps in the study of the interaction of variables, modeling and analysis of the responses with statistically valid results (Saxena et al., 2015). This approach helps an investigator to make

efficient exploration of a process system (Amiripour, et al., 2015). Several studies have been conducted using RSM for OD (Amiripour, et al., 2015, Saxena, et al., 2015, Maciel, et al., 2015, Gupta, et al., 2014, Patil, et al., 2014, Ganjloo, et al., 2014, Saxena, et al., 2012, Mercali, et al., 2011, Saxena, et al., 2009, Ozdemir, et al., 2008, El-Aouar, et al., 2006, Uddin et al., 2004)

This research aimed to assess the influence of solution temperature (40-60 oC), sugar concentration (40-60 oB), calcium lactate salt concentration (1-3%) and immersion time (120-180 min) of osmotic dehydration (OD) of pear, establish process conditions which are able to provide maximum water loss, weight reduction and Overall acceptability score (OAA) and minimum solid gain and water activity in the product and optimizing using response surface methodology. In addition, the osmotic dehydration kinetics at optimized conditions was also studied and fitted to different empirical models viz. Magee's model, Page's model and Azuara's model so as to generate the data to design the OD systems.

## 2. Materials and methods

### *2.1. Preparation of sample*

Fresh pears of uniform size were obtained from Agricultural Produce Marketing Committee (APMC), Azadpur market, New Delhi, India and were stored in the refrigerator before the use for the experiments. The fruits were washed using potable water, hand peeled with knife, de-seeded and vertically cut into four pieces. The average moisture content of pears was found to be 85.285% wet basis by oven drying method subjecting the uniform mash of fresh pears to 105 $^{o}$C for 5 hours (AOAC, 2000). The osmotic solutions in the range of 40-60 $^{o}$B were prepared by mixing food grade sucrose (amorphous refined sugar) and food grade calcium lactate salt in powder form (Universal laboratories, New Delhi, India) in the range of 1-3% w/w with calculated quantity of distilled water according to the experimental design. The conc. of sugar solutions was determined using a portable hand held refractometer (Omega RFH 101 & 201 of measuring range of 28-62% and 58-92%. In each of the experiments, fresh osmotic solution was used. All the experiments were done in triplicate and the average value was taken for calculations. The sample to solution ratio of 1:5 by weight was chosen to avoid significant dilution during OD (Sangamithra*et al.*, 2014, Uddin et al., 2004; Le Marguer, 1988).

### *2.2. Osmotic dehydration*

Sample of 50 g of pear slices were weighed and totally submerged into the osmotic solutions as per the experimental design. At each time of sampling (120-180 min), the pear slices were drawn out and quickly rinsed under a fast flowing stream of cold water, then gently blotted with adsorbent paper and weighed with an analytical balance (Schimadzu make) with an accuracy of $\pm$ 0.001g and the residual moisture and solid content after the osmosis was determined using the oven drying method (AOAC, 2000). Experiments were performed in triplicates and the average values were reported in order to average out any possible in accuracies. OD kinetics were analysed using the gravimetric equation (1-3) for the mass transfer parameters, water loss (WL), solid gain (SG) and weight reduction (WR) and were expressed in percentage of initial composition (Ozen*et al.*, 2002; Singh *et al.*, 2007; Fernandes and Rodrigues, 2008). The response surface methodology with Box-Behnken design using the software Design Expert Trial 7.0.0 version, Statease Inc., Minneapolis, USA was used to optimize the processing conditions process variables (Table 1) during OD of pears.

**Table 1.**Experimental variables and their levels used for Response Surface Methodology (Box Behnken Design) osmotic dehydration of Pear fruits

| | Symbols | -1 | 0 | 1 |
|---|---|---|---|---|
| Temperature ($^oC$) | A | 40 | 50 | 60 |
| Sugar Concentration ($^oB$) | B | 40 | 50 | 60 |
| Calcium salt (%) | C | 1 | 2 | 3 |
| Treatment time (min) | D | 120 | 150 | 180 |

These response variables were calculated using the data of weight and moisture content of each sample.

$$\% \text{ WL} = \frac{(W_0 - W_t) + (S_t - S_0)}{W_0} \times 100 \quad (1)$$

$$\% \text{ SG} = \frac{S_t - S_0}{W_0} \times 100 \quad (2)$$

$$\% \text{ WR} = \text{WL-SG} \quad (3)$$

where, $W_0$ is the initial weight of sample taken before OD, Wt is the weight of the sample after OD at the time t, $S_0$ is the initial weight of solids of the sample and $S_t$ is the weight of solids (dry matter) in the sample after OD at the time t.

*2.3. Water activity*

The equilibrium amount of water which is available for the hydration of materials is referred to aswater activity. If there is a value of unity, it indicates pure water, whereas zero indicates the total absence of any water molecule (bone-dry matter). $a_w$ of a sample only depends upon its water content and corresponding temp. Dew point equipment (Aqua Laboratory, Decagon CX-2, Decagon Devices Inc., Pullman, Washington, USA) was used for the determination of $a_w$ at 25 °C.

*2.4. Color measurements*

The color of the pear slices was measured using Labscan XE colorimeter (HunterLab, Inc., Reston, VA, USA) under D65 illuminating lamp conditions keeping the observer angle of $10^o$, after calibrating it with a white ceramic tile. The color values were expressed as *L*- value (lightness/ darkness), *a*- value (redness/greenness) and *b*- value (yellowness/ blueness) on the Hunter scale.

*2.5. Sensory evaluation*

Osmosed product was evaluated for color, taste, texture and its overall acceptability (OAA). Ten trained panelists conductedthe sensory evaluation using a nine point hedonic scale (9: excellent; 7: good; 5: acceptable (limit of marketability); 3: poor and 1: extremely poor) (Larmond, 1977). The samples were coded and were drawn from each level of the experimental design, and presented to the panelists randomly in a whitelight illuminated room and maintained at $25^oC$.

*2.6. Optimizationand statistical analysis*

Second order polynomial equation 4 was used to fit the experimental data which further described the combined effect of all the variables on the response Y and determined the interrelationship among the variables.

$$Y_i = ß_0 + ß_1X_1 + ß_2X_2 + ß_3X_3 + ß_4X_4 + ß_{11}X_1^2 + ß_{22}X_2^2 + ß_{33}X_3^2 + ß_{44}X_4^2 + ß_{12}X_1X_2 + ß_{13}X_1X_3 + ß_{14}X_1X_4 + ß_{23}X_2X_3 + ß_{24}X_2X_4 \quad (4)$$

where $Y_i$ is the predicted response for WL, SG, WR, *L*- value, *a*- value, *b*- value, $a_w$, and OAA score.$\beta_0$ is the estimated coefficient of the fitted response at the center point of the design, $\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$ are linear, $\beta_{11}$, $\beta_{22}$, $\beta_{33}$, $\beta_{44}$ quadratic and $\beta_{12}$, $\beta_{13}$,$\beta_{14}$, $\beta_{23}$, $\beta_{24}$ are interaction coefficients, respectively. Non linear statistical optimization procedure was used to fit the polynomials for various responses studied. The surface plots were used to show the fitted

polynomial equations so that the relationship between the response and experimental levels of each factor can be visualized and used to interpret the optimum conditions. Graphical optimization technique was used to find workable optimum conditions by fixing two of the variables at predetermined optimum level. Therefore, osmotic dehydration process was optimized on conditions giving maximum WL, WR and OAA score, minimum SG and $a_w$ and determines the levels of temp., sugar solution conc., Ca salt and immersion time in osmotically dried product.

## 3. Results and discussions

Preservation of pearby osmotic dehydration instead of conventional drying was done by development of high moisture stabilized pear pieces through an optimized process. OD over conventional drying is highly desirable and advantageous for a high quality product with improved shelf life.

### *3.1. Osmotic dehydration process*

Experiments were conducted for different combinations of the process variables viz. temp. of ternary solution , sugar conc., calcium salt conc. and immersion time and their responses WL, SG, WR, *L*- value, *a*- value, *b*- value, $a_w$and OAA score were obtained (Table 2). Optimization of the process was carried out as per the Box-benhken design of the response surface methodology. ANOVA was used to evaluate the significance of linear, quadratic and interaction effect of each variable on the responses. The sum of squares of all the responses were found to be significant ($p<0.01$). A second order polynomial model (Eq 8) explains the variability in responses with high coefficient of determination ($R^2> 0.90$) representing good fit (Table 3). The statistical lack of fit test for all responses was non-significant ($p>0.05$) and therefore, it conforms to the statistical methodology used and is applicable in the case of osmotic dehydration of pear slices (Khuri and Cornell, 1996; Saxena *et al.*, 2009). The combined effect of the two variables on any response for each fitted model was studied as the function of two independent variables. So, the software was used to develop three-dimensional plots of the response surface of variables while keeping the other two variables at centre value. Response surface plots were generated for each combination of variables keeping the other two variables at centre points as constant factor. Effects of variables on responses were explained by the plotted response surface figures.

### *3.2. Influence of variables on water loss*

The range of water loss varied from 14.86 to 28.33% (Fig 1a). Experiment no. 17, resulted in highest WL which corresponded to 50 $^oC$ temp., 50 $^oB$ sugar conc., 3% calcium salt conc. and 180 min immersion time while experiment no. 25 resulted in minimum WL which corresponded to 50 $^oC$ temp., 40 $^oB$ sugar conc., 2% calcium salt conc. and 120 min immersion time (Table 2).

**Table 2.** Experimental design and values of response variables for osmotic dehydration of Pear

| Experimental run | A:Temperature | B:Sugar Conc. | C: CaCl2 | D: Time | WL | SG | WR | *L*- value | *a* - value | *b* -value | aw | OAA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ($^oC$) | ($^oB$) | (%) | (min | (%) | (%) | (%) | | | | | |
| 1 | 40 | 50 | 2 | 120 | 17.46 | 5.7 | 11.81 | 69.55 | -3 | 17.506 | 0.736 | 6.83 |
| 2 | 50 | 50 | 3 | 120 | 19.55 | 6.89 | 12.69 | 60.81 | -3.12 | 17.546 | 0.652 | 8.01 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 | 50 | 60 | 3 | 150 | 23.27 | 7.5 | 15.71 | 48.18 | -3.21 | 17.371 | 0.691 | 7.94 |
| 4 | 40 | 40 | 2 | 150 | 17.35 | 6.53 | 10.87 | 68.11 | -3.22 | 17.085 | 0.671 | 7.35 |
| 5 | 50 | 50 | 1 | 120 | 18.37 | 6.45 | 11.97 | 64.59 | -2.94 | 17.371 | 0.823 | 8.22 |
| 6 | 40 | 50 | 2 | 180 | 25.47 | 8.27 | 17.26 | 52.78 | -2.31 | 17.452 | 0.799 | 7.19 |
| 7 | 50 | 60 | 2 | 120 | 17.69 | 5.91 | 11.75 | 54.23 | -3.26 | 17.597 | 0.723 | 8.26 |
| 8 | 50 | 50 | 2 | 150 | 25.71 | 8.11 | 17.55 | 44.34 | -3.52 | 17.25 | 0.843 | 8.4 |
| 9 | 60 | 50 | 2 | 180 | 27.97 | 8.68 | 19.33 | 46.56 | -1.99 | 17.536 | 0.688 | 6 |
| 10 | 50 | 40 | 2 | 180 | 23.01 | 8.19 | 14.78 | 47.08 | -2.8 | 17.435 | 0.7 | 8.07 |
| 11 | 50 | 60 | 1 | 150 | 21.53 | 8.03 | 13.45 | 51.01 | -3 | 17.196 | 0.724 | 7.95 |
| 12 | 60 | 50 | 1 | 150 | 22.92 | 7.69 | 15.17 | 58.46 | -2.53 | 17.114 | 0.605 | 5.76 |
| 13 | 50 | 50 | 2 | 150 | 25.73 | 8.13 | 17.57 | 44.14 | -3.5 | 17.27 | 0.753 | 8.42 |
| 14 | 60 | 50 | 3 | 150 | 24.87 | 8.11 | 16.71 | 54.98 | -2.71 | 17.254 | 0.789 | 6.9 |
| 15 | 60 | 60 | 2 | 150 | 22.54 | 7.23 | 15.36 | 52.18 | -2.67 | 17.276 | 0.849 | 6.72 |
| 16 | 50 | 50 | 1 | 180 | 26.42 | 9.16 | 17.31 | 48.48 | -2.22 | 17.345 | 0.788 | 7.23 |
| 17 | 50 | 50 | 3 | 180 | 28.33 | 9.55 | 18.85 | 43.09 | -2.45 | 17.6 | 0.692 | 8.42 |
| 18 | 50 | 50 | 2 | 150 | 25.81 | 8.21 | 17.65 | 45.74 | -3.42 | 17.35 | 0.575 | 8.5 |
| 19 | 40 | 50 | 1 | 150 | 21.2 | 7.38 | 13.78 | 65.73 | -2.86 | 17.023 | 0.675 | 6.91 |
| 20 | 50 | 50 | 2 | 150 | 25.8 | 8.2 | 17.64 | 45.74 | -3.43 | 17.34 | 0.688 | 8.49 |
| 21 | 60 | 40 | 2 | 150 | 20.22 | 6.71 | 13.56 | 61.09 | -2.8 | 17.176 | 0.821 | 6.53 |
| 22 | 50 | 40 | 3 | 150 | 19.99 | 8.05 | 11.88 | 56 | -3.42 | 17.236 | 0.827 | 8.54 |
| 23 | 60 | 50 | 2 | 120 | 19.16 | 5.9 | 13.29 | 63.43 | -2.66 | 17.455 | 0.808 | 6.95 |
| 24 | 50 | 40 | 1 | 150 | 18.66 | 6.72 | 11.89 | 62.53 | -3.2 | 16.981 | 0.778 | 7.57 |
| 25 | 50 | 40 | 2 | 120 | 14.86 | 5.22 | 9.6 | 65.2 | -3.37 | 17.404 | 0.842 | 8.21 |
| 26 | 40 | 50 | 3 | 150 | 22.34 | 7.77 | 14.51 | 59.75 | -3.09 | 17.313 | 0.849 | 6.76 |
| 27 | 50 | 50 | 2 | 150 | 25.73 | 8.13 | 17.57 | 44.14 | -3.5 | 17.27 | 0.84 | 8.42 |
| 28 | 40 | 60 | 2 | 150 | 21.16 | 6.76 | 14.45 | 57.29 | -2.95 | 17.335 | 0.85 | 6.91 |
| 29 | 50 | 60 | 2 | 180 | 26.32 | 8.26 | 18.01 | 38.71 | -2.5 | 17.593 | 0.842 | 7.78 |

The process variables which were expressed as the linear and quadratic terms had significant ($p<0.05$) effect on WL as shown from the analysis of variance.The values of the regression coefficient of the fitted second order polynomial was used for the interpretation of relationship between responses and variables. Itcan be seen that the effect on WL in descending order of the process variables on the basis of the values of regression coefficient are immersion time ($a_4$=4.20), sugar conc. of ternary solution ($a_2$=1.54), temp. of ternary solution (a1=1.06) and calcium salt conc. (a3=0.77). These results indicated that time of immersion and sugar conc. are more important variables influencing the water loss of pear slices in comparison to the temp. and calcium salt conc. The interaction of process variables, showed a significant effect on WL during OD ($p<0.05$)(Table3).

**Table 3.** Regression coefficients of the fitted second-order polynomials representing the relationship between

| Coefficients | WL (%) | SG (%) | WR (%) | L- value | a value | b value | $a_w$ | OAA |
|---|---|---|---|---|---|---|---|---|
| $a_0$ | 25.76 | 8.16 | 17.60 | 44.82 | -3.474 | 17.30 | 0.8446 | 8.45 |
| $a_1$ | 1.06* | 0.16* | 0.90* | -3.04* | 0.173* | 0.01* | -0.0263* | -0.26* |
| $a_2$ | 1.54* | 0.19* | 1.35* | -4.87* | 0.102* | 0.09* | -0.0063* | -0.06* |
| $a_3$ | 0.77* | 0.20* | 0.57* | -2.33* | -0.104* | 0.11* | 0.0239* | 0.24* |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $a_4$ | 4.20* | 1.34* | 2.87* | -8.43* | 0.340* | 0.01* | -0.0148* | -0.15* |
| $a_{12}$ | -0.37* | 0.07* | -0.45* | 0.48 | -0.035 | -0.04* | 0.0160* | 0.16* |
| $a_{13}$ | 0.20* | 0.01 | 0.20* | 0.63 | 0.013 | -0.04* | 0.0325* | 0.32* |
| $a_{14}$ | 0.20* | 0.05 | 0.15* | -0.03 | -0.005 | 0.03* | -0.0328* | -0.33* |
| $a_{23}$ | 0.10* | -0.47* | 0.57* | 0.93 | 0.002 | -0.02 | -0.0243* | -0.25* |
| $a_{24}$ | 0.12* | -0.16* | 0.27* | 0.65 | 0.048 | -0.01 | -0.0090* | -0.09* |
| $a_{34}$ | 0.18* | -0.01 | 0.21* | -0.40 | -0.013 | 0.02 | 0.0360* | 0.35* |
| $a_{11}$ | -1.74* | -0.60* | -1.12* | 10.09* | 0.483* | -0.05* | -0.1476* | -1.50* |
| $a_{22}$ | -3.75* | -0.80* | -2.97* | 4.05* | 0.032* | -0.03* | -0.0143* | -0.12* |
| $a_{33}$ | -1.14* | 0.22* | -1.39* | 5.55* | 0.238* | -0.07* | -0.0351* | -0.32* |
| $a_{44}$ | -1.50* | -0.42* | -1.05* | 3.16* | 0.505* | 0.24* | -0.0173* | -0.20* |
| $R^2$ | 0.9999 | 0.9986 | 0.9998 | 0.9947 | 0.9909 | 0.9893 | 0.9976 | 0.9976 |
| *Significant at 5% level | | | | | | | | |

WL increased with the increase in immersion time, temp., solution conc., in osmotic solution, while loss of water was also affected by calcium salt conc. It reveals that water loss increased more with the increased sugar conc. than in temp. of ternary solution. During the investigation, it was found that at the higher sugar conc. and process temp., the water loss was faster and also reduced the immersion time to reach the equilibrium.As temperature increases the membrane permeability increases, leading to swelling and plasticization of the cell membranes therefore favoring mass transfer from the tissue (Mercali*et al.,* 2012; Lazarides*et al.,* 1995).With the rise in temp., the viscosity of the solution decreases and the external resistance to mass transfer reduces allowing water and solute transport easier (Mercali*et al,*. 2012; Tonon*et al.,* 2007).Sugar having higher molecular weight than calcium lactate might have remained on the surface of the pear slices and may have allowed higher impregnation of calcium salt into the pear slices resulting in enhanced water loss. The sugar conc. and calcium salt have a synergistic effect on WL and increased the firmness of plant tissues as well (Pereira *et al.,* 2006, Ferrari *et al.,* 2010 Mavroudis*et al.,* 2012; Mercalli *et al.,* 2012 and Amiripour*et al.,* 2015). Similar results have also been reported for osmotic dehydration of cherry tomatoes by Derossi*et al.,* (2015), aloe vera gel cubes by Pisalkar*et al.,* (2011) and pumpkin by Mayor *et al.*, (2007). The possible damage caused to the cell membranes of the pear slices by the osmotic dehydration process may have been compensated by the calcium ions in the solution by cross linking the pectic polymers and a calcium fortified product has been obtained (Barrera *et al.,* 2004, Anino*et al.,* 2006 and Silva *et al.,* 2014).Three dimensional response surface was plotted for the WL. Fig. 1a shows the most significant interaction plot between process variables. It is selected on the basis of high correlation coefficient among all interaction of the variables. It shows that as temp. increases, WL increases. In the same way as the calcium salt conc. increases, WL increases.

The multiple regression equation at 5% level of significance and neglecting the non-significant terms provided a good fit for describing the relationship between the process variables and response. The uncoded process variable form of the developed model, is as follows:

$$WL = -185.34 + 1.89 * Temp. + 4.01 * Sugar\ Conc. + 2.89 * Calcium\ salt + 0.57 * Time - 0.004 * Temp. * Sugar\ Conc. + 0.02 * Temp. * Calcium\ salt + 0.0007 * Temp. * Time + 0.01 * Sugar\ Conc. * Calcium\ salt + 0.0004 * Sugar\ Conc. * Time + 0.0061 * Calcium\ salt * Time - 0.0174 * Temp^2 - 0.04 * Sugar\ Conc.^2 - 1.14 * Calcium\ salt^2 - 0.0017 * Time^2 \quad (1)$$

*3.3. Influence of variables on solid gain*

The range of SG varied from 5.22 to 9.55% (Fig 1b). Experiment no. 17, resulted in highest SG which corresponded to 50 $^{o}$C temp., 50 $^{o}$B sugar conc., 3% calcium salt conc. and 180 min immersion time while experiment no. 25 resulted in minimum SG which corresponded to 50 $^{o}$C temp., 40 $^{o}$B sugar conc., 2% calcium salt conc. and 120 min immersion time (Table 2). The process variables which were expressed as the linear and quadratic terms had significant ($p<0.05$) effect on SG as shown from the analysis of variance. The values of the regression coefficient of the fitted second order polynomial was used for the interpretation of relationship between responses and variables. Itcan be seen that the effect on SG in descending order of the process variables on the basis of the values of regression coefficient are immersion time ($a_4$=1.34), calcium salt conc. ($a_3$=0.20), sugar conc. of ternary solution ($a_2$=0.19), temp. of ternary solution (a1=0.16) and. These results indicated that time of immersion and calcium salt conc. are more important variables influencing the SG of pear slices in comparison to the temp. and sugar conc. (Table 3). SG increases more with the increase in sugar concentration than increase in temperature as $a_2$ is higher than $a_1$ while SG also increases with the increase in the osmosis time. The interaction of process variables, between temp. and sugar conc., sugar conc. and calcium salt conc., sugar conc. and immersion time showed a significant effect on SG during OD ($p<0.05$) (Table 3).

Three dimensional response surfaces were plotted for the SG. Fig. 1b shows the most significant interaction plot between process variables. It is selected on the basis of high correlation coefficient among all interaction of the variables. SG increased with increase in immersion time and sugar conc. as well as with increase in calcium salt and temp. (data not reported). Permeability of cell membrane is the reason for the solid gain. Permeability is lost due to the effect of the increase in temperature of the syrup and allows the solute to enter by losing its selectivity. The viscosity of the solution decreases at high temperatures and influences solid gain, because of the decrease in viscosity ultimately decreases the resistance to diffusion of solute into the sample tissue. Similar results have been reported for OD of cherry tomatoes by Derossi*et al.*, (2015), aloe vera gel cubes by Pisalkar*et al.*, (2011) and pumpkin by Mayor *et al.*, (2007). Due to high conc. difference between the pear slices and osmotic solution, SG in pear increased as the conc. of the osmotic solution increased (Mundada*et al.*, 2010). Cell plasmatic membrane allows solute to enter the pear slice because with the increase of the osmotic pressure gradient there is the loss of functionality of cell plasmatic membrane. Studies on osmotic dehydration of apple, pineapple (Sujata and Das, 2005), mango (Duduyemi, *et al.*, 2013) and cranberry (Shamaei, *et al.*, 2012) have shown similar results. There is non-significant effect ($p>0.05$) of temp. and calcium salt interaction and reveals that SG increases more with increase in temp. than increase in calcium salt conc.. The calcium salt conc. has non-significant ($p>0.05$) effect on the SG of pear with respect to immersion time. The multiple regression equation at 5% level of significance and neglecting the non-significant terms provided a good fit for describing the relationship between the process variables and response. The uncoded process variable formof the developed model is as follows:

$$SG = -50.58 + 0.55 * Temp. + 0.95 * Sugar\ Conc. + 1.66 * Calcium\ salt + 0.20 * Time + 0.000725 * Temp. * Sugar\ Conc. - 0.05 * Sugar\ Conc. * Calcium\ salt - 0.0005 * Sugar\ Conc. * Time - 0.006 * Temp.^2 - 0.008 * Sugar\ Conc.^2 + 0.22 * Calcium\ salt^2 - 0.0005 * Time^2 \quad (2)$$

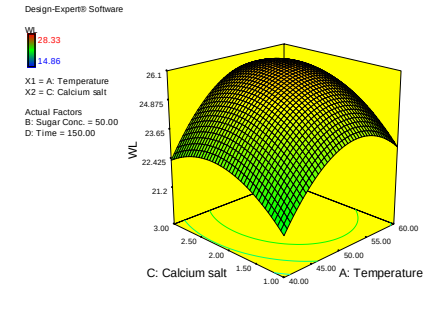
*3.4. Influence of variables on weight reduction*

The range of WR varied from 9.6 to 19.33% (Fig 1c). Experiment no. 9, resulted in highest WR which corresponded to 60 $^{o}$C temp., 50$^{o}$B sugar conc., 2% calcium salt conc. and 180 min immersion time while experiment no. 25 resulted in minimum WR which

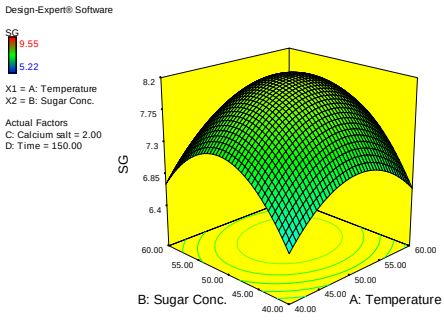
corresponded to 50 $^{o}$C temp., 40 $^{o}$B sugar conc., 2% calcium salt conc. and 120 min immersion time (Table 2). The process variables which were expressed as the linear and quadratic terms had significant ($p<0.05$) effect on WR as shown from the analysis of variance. The values of the regression coefficient of the fitted second order polynomial was used for the interpretation of relationship between responses and variables. Itcan be seen that the effect on WR in descending order of the process variables on the basis of the values of regression coefficient are immersion time ($a_4$=2.87), sugar conc. of ternary solution ($a_2$=1.35), temp. of ternary solution ($a_1$=0.90) and calcium salt conc. ($a_3$=0.57). These results indicated that time of immersion and conc. of ternary solution are more important variables influencing the WR of pear slices in comparison to the temp. and calcium salt conc. (Table 3). WR increases more with the increase in sugar concentration than increase in temperature as $a_2$ is higher than $a_1$ while WR also increases with the increase in the osmosis time. The interaction of process variables showed a significant effect on WR during OD ($p<0.05$) (Table 3). Three dimensional response surfaces were plotted for the WR. Fig. 1c shows the most significant interaction plot between process variables. It is selected on the basis of high correlation coefficient among all interaction of the variables. Other plot between immersion time versus temperature and immersion time versus sugar conc. and immersion time versus calcium salt conc. showed that immersion time is the most significant of all factors affecting weight reduction (data not reported).

With the passage of immersion time during OD, the tissue allows penetration of calcium salt having lower molecular weight and the sugar having more molecular weight might have got deposited on the surface of the tissue which may have posed an additional resistance to mass exchange and lowering the rates of WR. Similar trends have been reported by Ganjloo, *et al.*, 2014 for seedless guava and Jokic, *et al.*, 2007 for sugar beet. Due to the high concentration of the osmotic solution, the pear having soft tissues floated in the solution which created hindrances of contact between pear slices and osmotic solutions and thereby reducing the rate of mass transfer and ultimately the rate of WR also lowered. El-Aouar, *et al.* (2006) have also reported the reduction of mass transfer rates similarly during the osmotic dehydration of papaya. The multiple regression equation at 5% level of significance and neglecting the non-significant terms provided a good fit for describing the relationship between the process variables and response. The uncoded process variable form of the developed model, is as follows:

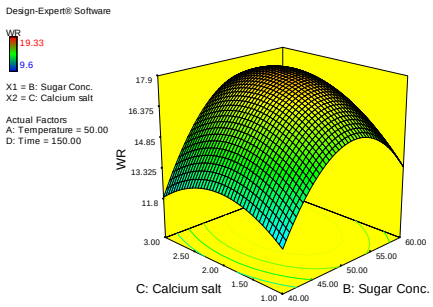
$$WR = -133.97 + 1.32 * Temp. + 3.08 * Sugar\ Conc. + 1.25 * Calcium\ salt + 0.36 * Time - 0.005 * Temp. * Sugar\ Conc. + 0.02 * Temp. * Calcium\ salt + 0.0005 * Temp. * Time + 0.06 * Sugar\ Conc. * Calcium\ salt + 0.0009 * Sugar\ Conc. * Time + 0.007 * Calcium\ salt * Time - 0.01 * Temp.^2 - 0.03 * Sugar\ Conc.^2 - 1.39 * Calcium\ salt^2 - 0.001 * Time^2 \quad (3)$$




**(a)**




**(b)**




**(c)**

**Figure 1.** Response surface plot for the effect of process variables on water loss (a), solid gain (b), and weight reduction (c) of pear slices

*3.5. Influence of variables on color values*

The range of *L*- value varied from 38.71 to 69.55 (Fig 2a). Experiment no. 1, resulted in highest *L*- value which corresponded to 40 $^{o}$C temp., 50 $^{o}$B sugar conc., 2% calcium salt conc. and 120 min immersion time while experiment no. 29 resulted in minimum *L*- value which corresponded to 50 $^{o}$C temp., 60 $^{o}$B sugar conc., 2% calcium salt conc. and 180 min immersion time (Table 2). The process variables which were expressed as the linear and quadratic terms had significant ($p<0.05$) effect on *L*- value as shown from the analysis of variance. The values of the regression coefficient of the fitted second order polynomial were used for the interpretation of relationship between responses and variables. It can be seen that the effect on *L*- value in descending order of the process variables on the basis of the values of regression coefficient are immersion time ($a_4$=-8.43), sugar conc. of ternary solution ($a_2$=-4.87), temp. of ternary solution ($a_1$=-3.04) and calcium salt conc. ($a_3$=-2.33). These results indicated that time of immersion and conc. of ternary solution are more important variables influencing the *L*- value of pear slices in comparison to the temp. and calcium salt conc. (Table 3). The interaction of process variables showed a non-significant effect on *L*- value during OD ($p<0.05$) (Table 3).

Fig. 2a describes the surface plot for the effect of pretreatment variables calcium salt conc. and sugar conc. at centre values of time and temp. on *L*- value of Pear slices. It is selected on the basis of high correlation coefficient among all interaction of the variables. It shows that *L*- value decreased with increase in sugar conc. and calcium salt conc.other significant plot between temp. and calcium salt conc. showed that as temp. and calcium salt conc. increased the *L*- value increased (data not reported). As the same time the surface plot between variables and immersion time showed horse saddle plot. Saxena *et al*. (2015) reported similar result for jackfruit bulb that visual colour is mainly a function of blanch. sol. conc. *L*- value also increased with increase in immersion time for osmosis and increase in calcium salt conc. of osmotic solution. The reason could be attributed to the impregnation of calcium salt and inward movement of solutes in the pear slice. Similar results have been reported that calcium salt conc. increased the visual colour by enhancing it. (Saxena *et al*., 2015). The multiple regression equation at 5% level of significance and neglecting the non-significant terms provided a good fit for describing the relationship between the process variables and response. The uncoded process variable form of the developed model, is as follows:

$$L\text{- value} = 624.80 - 10.74 * \text{Temp.} - 5.28 * \text{Sugar Conc.} - 30.27 * \text{Calcium salt} - 1.41 * \text{Time} + 0.1 * \text{Temp.}^2 + 0.04 * \text{Sugar Conc.}^2 + 5.55 * \text{Calcium salt}^2 + 0.004 * \text{Time}^2 \quad (4)$$

The range of *a*- value varied from -1.99 to -3.52 (Fig 2b). Experiment no. 8, resulted in highest *a*- value which corresponded to 50 $^{o}$C temp., 50 $^{o}$B sugar conc., 2% calcium salt conc. and 150 min immersion time while experiment no. 9 resulted in minimum *a*- value which corresponded to 60 $^{o}$C temp., 50 $^{o}$B sugar conc., 2% calcium salt conc. and 180 min immersion time (Table 2). The process variables which were expressed as the linear and quadratic terms had significant ($p<0.05$) effect on *a*- value as shown from the analysis of variance. The values of the regression coefficient of the fitted second order

polynomial were used for the interpretation of relationship between responses and variables. It can be seen that the effect on *a*- value in descending order of the process variables on the basis of the values of regression coefficient are immersion time ($a_4$=0.340), temp. of ternary solution ($a_1$=0.173), calcium salt conc. ($a_3$=-0.104) and sugar conc. of ternary solution ($a_2$=0.102),. These results indicated that time of immersion and temp. of ternary solution are more important variables influencing the *a*- value of pear slices in comparison to the sugar conc. and calcium salt conc. (Table 3).

The interaction of process variables showed a non- significant effect on *a*- value during OD ($p<0.05$) (Table 3). Fig. 2b describes the surface plot for the effect of process variables time and sugar conc. at centre values of temp.and calcium salt conc. on *a*- value of pear slices. The figure was selected on the basis of highest value of regression coefficient of interaction variables. It shows that *a*- value increased with the increase in immersion time and with the increase in sugar conc. The reason of increase in *a*- value with immersion time could be attributed to increase in solid content in the pear slice. Similar result has been reported that use of calcium salt increased the visual color of the products (Saxena *et al*., 2015). The multiple regression equation at 5% level of significance and neglecting the non-significant terms provided a good fit for describing the relationship between the process variables and response. The uncoded process variable form of the developed model is as follows:

a value = 20.32 -0.45 * Temp. -0.03 * Sugar Conc. -1.07 * Calcium salt -0.16 * Time + 0.005 * Temp.$^2$ + 0.0003 * Sugar Conc.$^2$ + 0.24 * Calcium salt$^2$+ 0.0006 * Time$^2$ (5)

The range of *b*- value varied from 16.981 to 17.6 (Fig 2b). Experiment no. 17, resulted in highest *b*- value which corresponded to 50 $^o$C temp., 50 $^o$B sugar conc., 3% calcium salt conc. and 180 min immersion time while experiment no. 24 resulted in minimum *b*- value which corresponded to 50 $^o$C temp., 40 $^o$B sugar conc., 1% calcium salt conc. and 150 min immersion time (Table 2). The process variables which were expressed as the linear and quadratic terms had significant ($p<0.05$) effect on *b*- value as shown from the analysis of variance. The values of the regression coefficient of the fitted second order polynomial were used for the interpretation of relationship between responses and variables. It can be seen that the effect on *b*- value in descending order of the process variables on the basis of the values of regression coefficient are calcium salt conc. ($a_3$=0.11), conc. of ternary solution ($a_2$=0.09), immersion time ($a_4$=0.01), temp. of ternary solution ($a_1$=0.01), and These results indicated that calcium salt conc. ($a_3$=0.11) and sugar conc. of ternary solution ($a_2$=0.09)are more important variables influencing the *b*- value of pear slices in comparison to the sugar conc. and calcium salt conc. (Table 3).The interaction of process variables showed a non- significant effect on *a*- value during OD ($p<0.05$) (Table 3).Fig. 2c describes the surface plot for the effect of pretreatment variables sugar conc. and temp. at centre values of immersion time and calcium salt conc. on *b*- value of pear slices. It shows that as temp. and sugar conc increased the *b*- value increased. The figure was selected on the basis of highest value of regression coefficient of interaction variables. The multiple regression equation at 5% level of significance and neglecting the non-significant terms provided a good fit for describing the relationship between the process variables and response. The uncoded process variable form of the developed model is as follows:

b value = 19.65 + 0.06 * Temp. + 0.07 * Sugar Conc. + 0.58 * Calcium salt -0.085 * Time - 0.0004 * Temp. * Sugar Conc. -0.004 * Temp. * Calcium salt + 0.0001 * Temperature * Time - 0.000490417 * Temp.$^2$ -0.0003 * Sugar Conc.$^2$ - 0.07 * Calcium salt$^2$ + 0.0003 * Time$^2$

(6)

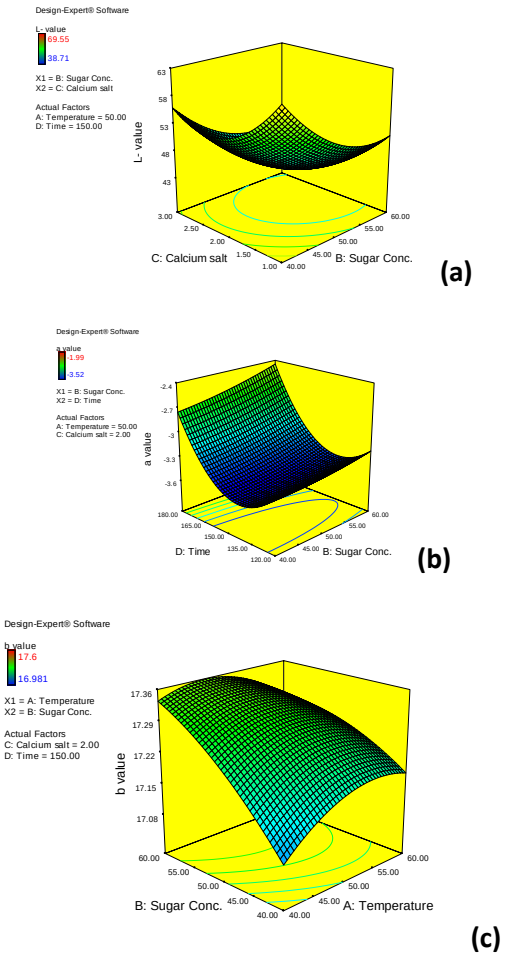



**Figure 2.** Response surface plot for the effect of process variables on *L*- value (a), *a*- value (b), *b* value (c) of pear slices

*3.6. Influence of variables on water activity*

The range of water activity ($a_w$) varied from 0.576 to 0.855 (Fig 2b). Experiment no. 28, resulted in highest $a_w$ which corresponded to 40 $^oC$ temp., 60 $^oB$ sugar conc., 2% calcium salt conc. and 150 min immersion time while experiment no. 18 resulted in minimum $a_w$ which corresponded to 50 $^oC$ temp., 50 $^oB$ sugar conc., 2% calcium salt conc. and 150 min immersion time (Table 2). The process variables which were expressed as the linear and quadratic terms had significant ($p<0.05$) effect on $a_w$ as shown from the analysis of variance. The values of the regression coefficient of the fitted second order polynomial were used for the interpretation of relationship between responses and variables. It can be seen that the effect on $a_w$ in descending order of the process variables on the basis of the values of regression coefficient are temp. of ternary solution ($a_1$=-0.0263), calcium salt conc. ($a_3$=0.0239), immersion time ($a_4$=-0.0148) and sugar conc. of ternary solution ($a_2$=-0.0063). These results indicated that temp. of ternary solution ($a_1$=-0.0263) and calcium salt conc. ($a_3$=0.0239) are more important variables influencing the $a_w$ of pear slices in comparison to the sugar conc. and calcium salt conc. (Table 3). The interaction of process variables showed a significant effect on *a*-value during OD ($p<0.05$) (Table 3). Fig. 2c describes the surface plot for the effect of pretreatment variables immersion time and calcium salt conc. at centre values of immersion time and temp. on $a_w$ of pear slices. It shows that as time and calcium salt conc. increased the $a_w$ decreased. The figure was selected on the basis of highest value of regression coefficient of interaction variables. These results indicated that $a_w$ the decreased with increase in immersion time for osmosis and increase in calcium salt in comparison to sugar conc. and temp. of osmotic solution. The reason could be attributed to the impregnation of calcium salt in comparison to sugar due to it lower molecular weight than sugar and lowering the $a_w$ of the sample (Table 3). Restriction of gain in sugar by the addition of calcium salt have been observed by various researchers; for guavas which were osmotically dehydrated in maltose solutions but not for papaya in sucrose solutions by Pereira *et al*., 2006 and the reason reported for the same was the specific tissue structure of each fruit. In another research on osmotic dehydration of apples, solute gain reduced by the addition of 0.6% calcium lactate to solution, and reason reported for the same was a reduction in cell wall porosity (Mavroudis*et al*., 2012). Silva *et al*., 2014, conducted experiments for pineapple by OD and also attributed the reason for limited

transfer of sucrose into pineapple tissue to the present pectin and enzymes in the fruit. Pectin methyl esterase, an important enzyme present in the pineapple, hydrolyses the pectin methyl esters (Silva *et al*., 2011a and Silva *et al*., 2011b) and generates carboxyl groups that interact with calcium (Guillemin *et al*., 2008) and promotes the cross-linking of pectin polymers which repairs the cell walls (Anino*et al*., 2006). The cuts and injuries to the tissue releases enzymes which reacts with the calcium forming the calcium pectate around the cut surfaces and would act as a partial barrier to the diffusion of larger molecules such as sucrose in to the tissue (Barrera *et al*. 2009; Silva *et al*., 2014). This shows that loss of water also influences water activity. Water activity is a critical indicator for shelf life of the pear because of microbial stability (Guiamba*et al*., 2016). Three dimensional response surfaces for $a_w$ were also plotted. Figure 3a shows the surface plot with the most significant ($p<0.05$) interaction for the effect of process variables of temp. and time at centre values of sugar conc. and calcium salt conc. on $a_w$ of pear slices. as the temp. and time increases, $a_w$decreases which is the desirable effect for the stability of the final product.The multiple regression equation at 5% level of significance and neglecting the non-significant terms provided a good fit for describing the relationship between the process variables and response. The uncoded process variable form of the developed model, is as follows:

aw = -3.79 + 0.15 * Temp. + 0.02 * Sugar Conc. - 0.06 * Calcium salt + 0.01 * Time + 0.0002 * Temp. * Sugar Conc. + 0.003 * Temp. * Calcium salt - 0.0001 * Temp. * Time - 0.002 * Sugar Conc. * Calcium salt – 0.00003 * Sugar Conc. * Time + 0.0012 * Calcium salt * Time -0.002 * Temp.$^2$ -0.0001 * Sugar Conc.$^2$ -0.035 * Calcium salt$^2$ – 0.00001 * Time$^2$ (7)

### *3.7. Influence of variables on overall acceptability score*

The overall acceptability (OAA) score profile for different combinations of process variables varied from 5.76 to 8.54 (Fig. 3b) for different combinations of treatments. Among them, combination no. 12 resulted in the lowest OAA score corresponding to 60ºC temp., 50ºB sugar conc., 1% calcium salt and 150 min immersion time while combination no. 22 gave the highest score corresponding to 50 ºC temp., 40ºB sugar conc., 3% calcium lactate salt and 150 min immersion time (Table 2). The experimental variables temp., sugar conc., calcium salt and immersion time were found to have significant ($p<0.05$) effect on OAA score profile. The analysis of variance of the responses indicated that all linear terms as well as quadratic terms have significant effect ($p<0.05$) on OAA score. To understand the positive effects in ascending order of the variables on OAA score, the values of the regression coefficients of the fitted second-order polynomial representing the relationship between the responses and variables can be used for interpretation. The magnitude of coefficients indicates maximum positive effect of temp. ($a_1$=-0.26), calcium salt conc. ($a_3$=0.24), immersion time ($a_4$=-0.15), followed by sugar conc. ($a_2$=-0.06) (Table 3). These results indicate that OAA score was affected by calcium salt conc. and immersion time of the pear slices. Saxena *et al.*(2012) reported sensory attributes of jackfruits bulbs to be effected by calcium salt conc. and immersion time. Figure 3b shows the significant ($p<0.05$) interaction surface plot for the effect of pretreatment variables sugar conc. and temp. at centre values of immersion time and calcium salt conc. on OAA score of pear slices. The multiple regression equation at 5% level of significance and neglecting the non-significant terms provided a good fit for describing the relationship between the process variables and response. The uncoded process variable form of the developed model, is as follows:

OAA score = -38.58 + 1.49 * Temp. + 0.13 * Sugar Conc. -0.60 * Calcium salt + 0.11 * Time + 0.002 * Temp. * Sugar Conc. + 0.03 * Temp. * Calcium salt - 0.001 * Temp. * Time - 0.02 * Sugar Conc. * Calcium salt -0.0003 * Sugar Conc.* Time + 0.01 * Calcium salt *

Time -0.02 * Temp.$^2$ - 0.001 * Sugar Conc.$^2$ - 0.32 * Calcium salt$^2$ -0.0002 * Time$^2$ (8)

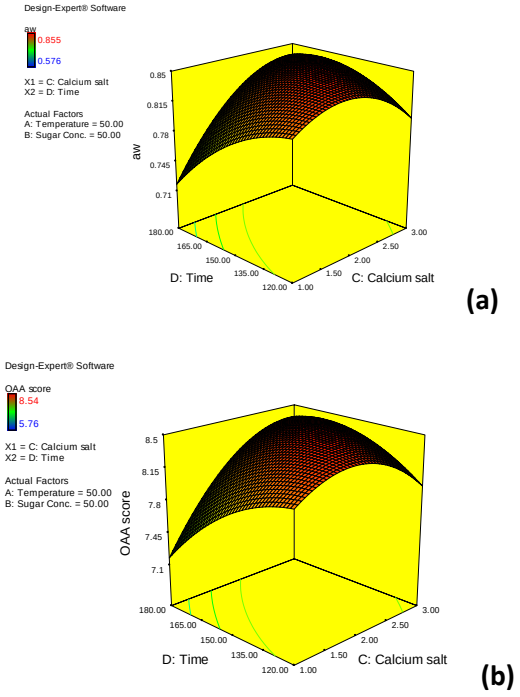



**Figure 3.**Response surface plot for the effect of process variables on water activity (a) and overall acceptability (b) of pear slices

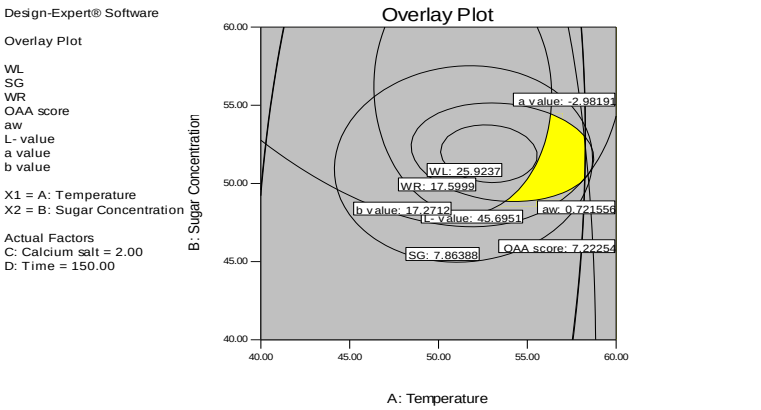
*3.8. Optimization of osmotic dehydration pre-treatment*

Maximization goal was kept for WL, WR and OAA score while minimization goal was kept for SG and $a_w$ to obtain optimum conditions for OD for pear slices. The importance of variables were maximum for WL, WR, OAA score, $a_w$, *L*- value, *a*- value and *b*- value. Specified optimum dehydration conditions, were determined by fitting second order polynomial models obtained in the study, for each response. The regression models were only valid in the selected experimental domain. Therefore, the criteria for optimization were selected based on different parameters which included economical and product quality related attributes (Eren and Kaymak-Ertekin, 2007, Noshad*et al*, 2012).The optimum covering criteria deduced from the study was 59.57$^o$C for temp., 53.73$^o$B for sugar conc., 2.27% calcium salt conc. and 151.9 min immersion time by applying the desirability function method.

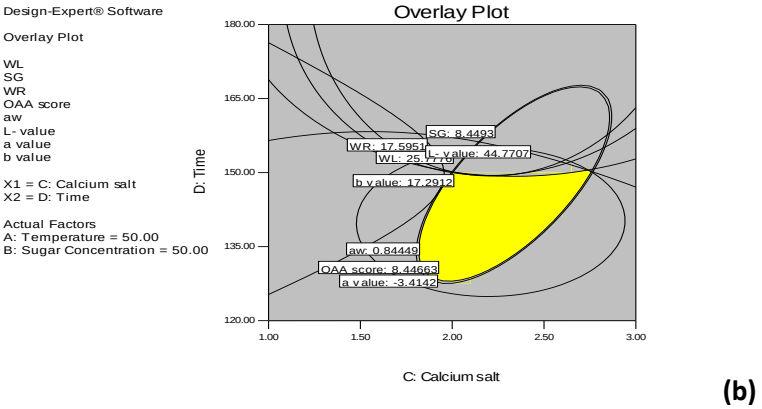
The graphical method of optimization was also adopted to deduce the workable range for optimized conditions. The effects whether main or interactive of the independent treatments on different responses were illustrated by the contour plots. In the set of four treatments, the contours were plotted between the two treatments while the other two were kept constant. The overlay plot for temp. and sugar conc. with calcium salt conc. and immersion time as its central value is described in Fig 4a and the overlay plot for calcium salt conc. and immersion time with temp. and sugar conc. as its central value is described in Fig 4b. The optimal zone for different combinations of treatments is represented by the shaded area highlighted within the overlay plots. The optimal ranges drawn from the overlay plat were found to be 54.3-59.1$^o$C temp. and 48.3-54.5$^o$B sugar conc. (Fig 4a). In the same way, the overlay plot between time and calcium salt conc., the optimal ranges from the overlay plot were found to be 127-150 min immersion time and 1.9-2.85 percent of calcium salt conc. (Fig 4b). The graphically and numerically obtained optimized conditions were in proximity. The optimization process was useful in ensuring the optimal OAA of the osmo-processed product.

*3.9. Verification of the final models*

The models obtained with the derived optimum conditions were further investigated under predictive optimum conditions. It was found that the predicted models were comparable to the experimental model shown in Table 4. This indicates that the second order polynomial model could be used to predict quality of osmotically dehydrated pear at different levels of temp., sugar conc., calcium salt and immersion time chosen as variable factors for the osmotic dehydration of pear.




**(a)**




**(b)**

**Figure 4.**Superimposed contours plot for the response variables of pear slices at varying temp. and sugar conc. (a), and at varying time and calcium salt conc. (b)

**Table 4.** Optimized independent variables and predicted and experimental

| **Variables** | Optimum condition | |
|---|---|---|
| Temperature ($^{o}$C) | 59.57 | |
| Sugar ($^{o}$B) | 53.73 | |
| Calcium lactate (%) | 2.27 | |
| Treatment time (min) | 151.9 | |
| Response | Predicted value | Actual value |
| WL (%) | 25.56 | 25.33±0.05 |
| SG (%) | 7.85 | 7.79±0.15 |
| WR (%) | 17.72 | 17.30±0.03 |
| L- value | 49.58 | 48.95±0.05 |
| a value | -2.82 | -2.79±0.05 |
| b value | 17.29 | 17.58±0.05 |
| Water activity aw | 0.693 | 0.71±0.05 |
| OAA score | 6.92 | 6.87±0.25 |

## 4. Conclusions

Osmotic dehydration was optimized by evaluating the effect of the sugar conc., temp., Ca salt conc., immersion time in an aqueous ternary solution on the water loss, solid gain, weight reduction, OAA score, water activity, and hunter color values such as *L*- value, *a*- value and *b*- value established using the response surface methodology (RSM). The process kinetics under the optimized conditions was also modeled. Sugar conc. was the most significant factor among all to affect the water loss and solid gain in the sample. Sugar gain and calcium impregnation were significantly affected by sugar and calcium lactate conc. Optimized conditions for the osmotic dehydration were process temperature 59.57$^{o}$C, Sugar concentration 53.73$^{o}$B, calcium salt 2.27 % and osmotic treatment time of 151.9 min were indicated during the course of study using the RSM. Although the optimal conditions for the osmotic dehydration process of pear was defined at an immersion time of 151.9 min using the RSM, the osmotic dehydration kinetics showed that process time of about 151.9 min will favor WL greater than 25.56% while keeping SG at approximately 7.85 % (Fig 6). Upper limit of the contact time according to experimental design was 180 min., however, it was observed that longer contact time up to 240 min were used to study the OD kinetics. Thus, OD was an effective pretreatment to obtain shelf stable pear slices with better overall characteristics. More detailed studies about the influence of calcium salt on the tissue micro-structure would be necessary to explain the changes in the mass transport studied in the present work.

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# INNOVATIVE TECHNOLOGICAL PROCESS FOR EMULGATED PATE PRODUCTION OUT OF FISH PROCESSING BY-PRODUCTS

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**ABSTRACT**

The rise in consumption of fish and fish products has led to the generation of high amounts fish processing by-products and there is an urgent need to convert this nutritious by-product into a value-added product that is suitable for human consumption. In this investigation, an innovative technological process has been developed to produce a pate out of fish processing by-products. An emulsifier, containing 59% water, 33% vegetable oil and 8% Tari Combi Pate emulsifying mix, was added to the homogenized smoked sprat heads along with salt, onion, phosphate blend, vegetable oil, vinegar, wheat fibre, and spices and thoroughly mixed to form a pate. The pate was packaged in tin cans or mason jars and sterilized in an industrial autoclave. This process proved to be successful based on microbial and organoleptic testing of the pate. This implies that this process of manufacturing fish waste pate has a potential to provide an alternative economic solution and expand the product assortment offered by fish processing companies.

## 1. Introduction

Fish wastes contribute to a significantly large percentage of the production volumes in the fish processing industries and hence it becomes vital to either dispose it safely or convert it into a value-added product. The former option is not encouraged due to its undesirable impact on the environment, moreover, it should not be wasted given its nutritional potential (Saranya et al., 2016). Value addition to fish wastes is an attractive alternative as it creates additional economic incentives to the manufacturer and ensures sustainability. Fish wastes have found several applications in the food and feed industry. Fish protein and protein concentrates, fish gelatine, nutraceutical ingredients, fish oils, natural pigments, collagen and chitosan are few of the compounds that are being extracted from fish waste. Research has shown that fish wastes are a source of such valuable biomolecules as enzymes, anti-microbial and antitumor compounds (Jayathilakan, et al., 2012). Utilizing the fish waste to fortify other food products is another option. Recently, a flaxseed and cinnamon cookie was enriched with cooked Nile tilapia fish bones was developed. This product was rich source of Calcium, Potassium, Iron and Omega 3 fatty acids (Abdel-Moemin, 2015).

Sprats, *Sprattus sprattus*, is a small herring-like, marine fish found in European waters especially in Irish Sea, Black Sea, Baltic Sea and the Sea of the Hebrides. Sprats can be canned, salted, grilled or fried. The canned sprats (usually smoked) or “Sprats in oil” are consumed in many North European countries and Latvia is one of the biggest producers and exporters of this commodity. Currently, the production of this sector is exported to 60 countries. Latvian fishing industry companies are successfully

penetrating new markets and have begun to export canned fish to such exotic markets as, for instance, Japan (Can you give a reference here).

Sprats are rich in long chain polyunsaturated fatty acids, both eicosapentaenoic acid C20:5 n-3 (EPA) and docosahexaenoic acid C22:6 n-3 (DHA), which are known as healthy fats because of their health benefits (Stołyhwo, et al., 2006). Like other fish, Sprats are also rich in protein, vitamins and minerals. The utilization of the wastes (usually smoked heads) generated by the canned Sprats industry should be promoted in order to reduce waste disposal issues, increase profitability of the industry and develop novel and nutritious food products.

Several fish pates are sold across the globe today, most of them are made from fresh or smoked fish flesh. Few attempts were made to utilise fish wastes for the production of fish pate. In one such study, Cachapinta pulp, a waste product of the filleting industry, was used to produce an edible pate (Lobo et al., 2015). The objective of the present study is to develop an innovative pate formulation and process using the heads of smoked sprats as raw material and access the final product for its sensory acceptability.

## 2. Materials and methods

### 2.1. Materials

The fish processing waste, i.e. the heads of Sprats (from in-house laboratory, Piejūra Ltd., Nīca, Latvia) were used to make the pate. Apple cider vinegar (Bajoriškių), Semolina (Valdo, Voldemārs, Latvia), Pea flour (Fasma, Latvia), Soy Isolate (Sojavit, Olimp, Poland), salt, black pepper and vegetable oil (sunflower oil) was procured from the convenience store. The phosphate blend and emulsifier used were TARI® P 22 and TARI COMBI Pate, respectively. The filler for the pate was VITACEL® Wheat Fiber WF 400 supplied by J. Rettenmaier & Söhne GmbH & Co. KG, Germany. The spice blend used was Fischburger by Frutarom Savory Solutions Austria GmbH.

### 2.2. Pre-processing of the fish heads

Washed and sorted fish were impaled on spikes through the gills and smoked, after which the fish were decapitated. As a result, the fish carcasses fell off while the heads remained on the spikes. These heads were the raw materials used to make the pate. To reduce the heterogeneous matter in the final product, two methods were employed, namely, boiling and acid treatment. In the boiling method, fish heads were boiled in water (fish:water ratio 1:2) for 5 min. The water was drained off and the fish heads were then used to make the pate. The acid treatment method involved adding 6% apple cider vinegar to fish heads at vinegar:fish ratio 7:100 during the pate preparation, along with the other additives.

### 2.3. Making of the oil emulsion/emulsifier

The oil emulsion is made according to the following ingredient proportions (%w/w): vegetable oil – 33%, water – 59% and emulsifier TARI COMBI Pate – 8%. Initially, water was poured in Bowl Chopper (Talsa - K15e) with chopping speed of 2840 RPM, followed by the emulsifier (TARI COMBI Pate). Vegetable oil was gradually added and allowed to mix (via the cutting action of blade rotation) until a homogenous texture was achieved.

### 2.4. Making of the pate

After pre-processing, the fish heads were ground to a paste like texture in the Bowl Chopper (Talsa - K15e) with chopping speed of 2840 RPM. Initial trials were carried out by combining the fish head paste with various concentrations of water, salt, onion, phosphate blend, vegetable oil, vinegar, wheat fibre, oil emulsion, soy isolate, pea

flour, semolina, spice blend and black pepper. The mixtures were thoroughly mixed in the same cutter to form a pate.

### 2.5. Packaging and thermal processing of the pates

The pate was poured into tin cans or mason jars and sterilized in industrial autoclave (Zirbus Technology HST-Series Chamber volume 200l). The sterilization program was set to product temperature of 121.1°C for 2 hrs 12 min.

### 2.6. Microbial Analysis

Microbial testing was conducted 2 weeks after autoclaving the pate in order to determine the presence of coliforms, lactobacillus and sulphite-reducing clostridia. The pate was also tested for yeast and moulds.

### 2.7. Nutritional analysis

The energy value and macro nutrient content of pate was calculated using a tabular method, in which, protein, fat and carbohydrates contents of the individual components of the product were used to calculate the nutritional value based on their percentage content in the formulation.

### 2.8. Organoleptic analysis

Pate samples were evaluated for organoleptic attributes like colour, aroma, texture, taste, aftertaste, and appearance. A set of 19 individuals, within the age group of 22 to 42 years, were asked to evaluate the above attributes on a 5-point hedonic scale (1 implies not good at all while 5 implies excellent). The results were analysed, and a spider chart was drawn. A multi-variate analysis of variance (MANOVA) was conducted to evaluate the null hypothesis that there are no differences in respondents' opinions regarding organoleptic parameters of 4 pate samples (N=19).

## 3. Results and discussions

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Pate, traditionally made from foie gras (or duck liver) and consumed mainly by the French and Danes (Huda, et al., 2011), is now available in many parts of the world. With palates accepting new tastes, there is immense potential for development of novel food products in the pate format, with nutritional and economic benefits to consumers and manufacturers, respectively. Fish, as such, is highly nutritious commodity and much of the waste from the fish processing industry is converted to animal feed or discarded. Fish wastes, especially from smoked fish, are not utilised to its full potential owing to the smoky flavour and dry texture. In this study, a process was developed to convert the fish heads of smoked sprats into edible pate.

### 3.1. Pre-processing and pate preparation

The heads of smoked sprats are considerably different from heads of fresh or frozen sprats. Due to the smoking process, the fish heads become dry, get saturated with smoke, lose elasticity, and the eyes harden. Unlike the heads of fresh or frozen sprats which can be easily cut in the cutter until the mass is homogeneous, if smoked heads are continuously cut in the laboratory cutter, the heterogeneous matter, i.e. eyes, jaw and gill particles, may not undergo size reduction. If the pate is made from heterogeneous matter, presence of the solid matter will considerably worsen the organoleptic parameters of the pate. The heterogeneous matter in the pate would be present in any cutting process since small particles (eyes, gills) of smoked fish heads are not crushed by the cutter blades since they are light and move freely between the blades and the bottom portion of the cutter tank.

There are several approaches available to homogenize the difficult to cut food

materials. Studies (Shimosaka, et al., 1998) indicated changes in physical properties and composition of fish bones (raw and steamed) when cured in acetic acid. Fish bones quickly softened due to elution of bone minerals and the effect was accelerated if the bones were steamed. In this study, two methods were implemented. First, boiling softened the fish heads as well as reduced the smell and smoky flavour. This pre-treatment considerably improved both, the organoleptic properties of the final product and the degree of homogenization of fish heads by the cutter. The other treatment was a chemical one in which the fish heads were treated with 6% apple cider vinegar. The acetic acid in vinegar helps to decalcify the bone tissue and solubilize collagen (Nagai & Suzuki, 2000) thus softening the fish heads as well as hydrating them. According to the results of further degustation of the finished pates, such amount of acetic acid did not cause excessive product acidity. Moreover, under the canned pate technology, canned pates, prior to being put on the market, are held in the finished product storage area for two weeks during which the process of decalcification continues.

Smoked heads contain high amounts of dry matter; therefore, a finely cut mixture cannot serve as the only ingredient. In the meat processing industry, pates are made from raw materials with high levels of fats and connective tissue, i.e. meat scraps, cheeks, fatty pig skin, etc., since meat processing enterprises do not usually suffer from the lack of such raw materials. Production of healthier pates by replacing pork backfat by oil combinations of olive oil, linseed oil and fish oil as well as konjac gel are reported (Delgado-Pando et al., 2011). At the fish processing plants, raw flesh materials rich in fats are not available and purchasing thereof is economically and technologically useless. Therefore, to make the pate, oil emulsion based on vegetable oil, water and emulsifiers was used. Vegetable oil is always used in the production of sprats, mayonnaise and making of the sauce for laminaria, and enterprises can buy it at low wholesale prices. Vegetable oil can easily be stored in usual storage areas and used in pate production. The proportion of said ingredients may be changed within a relatively wide range depending on the desirable emulsion texture, economic indicators (the emulsion is more expensive than the heads and forms a considerable part of the pate's production cost) and further use of the emulsifiers (for instance, isolated soy proteins). A considerable reduction in the dosage of the emulsifier may cause separation of the pate in cans or jars during sterilisation and storage as well as cause separation of the stock fatty substances which will worsen the organoleptic properties of the product. While making the emulsion it is extremely important to add the oil slowly to the water-emulsifier mix. If the oil is added quickly it may possibly cause emulsion spoilage and separation thereof right in the cutter tank or soon after cutting is finished.

To make the pate, a number of peculiarities were taken into account. From times immemorial, a pate has been regarded as a cheap product due to the low costs; therefore, this study aimed to avoid using expensive ingredients. Since smoked heads have a distinct flavour and smoke aroma, ingredients having a distinct and spicy flavour (onion, garlic, black pepper, etc.) were used rather than mild spices. A ready to use spice blend was also used in the initial trials but was considered uneconomical and to eliminate the dependency on supplier it was replaced with salt and pepper. Phosphate blends and salt were used to increase the water-binding capacity of fish raw materials and moisture retention. Filler were used to get intended texture of the pate. It was taken into account that collagen would gelatinise in the can/jars during sterilisation of the pate

and viscosity of the mass would increase; therefore, this factor was kept in mind to prevent the pate from becoming too thick. The finished product temperature was maintained between 12°C and 15°C before packaging to avoid microbial or biochemical degradation. It was observed that only a carefully configured and adapted high-speed cutter with sharp blades could be used for all stages of the pate production and nothing else was required. Four pate samples with good characteristics were identified after numerous initial trials and their composition is listed in the Table 1.

### 3.2. Packaging and thermal processing of the pates

The mode of sterilisation depends largely on the autoclaves available at the enterprise, and the duration of sterilisation depends on the initial microbial contamination of the fish heads, storage time and conditions, product acidity (mainly because of the vinegar added), the initial temperature of the pate prior to pre-packaging (making of the mass should be finished at the temperature not higher than +15°C), volume and shape of the can (the more its volume, the longer sterilisation time), container material (glass jars heat up slower than tin cans). Can/jar rotation during thermal processing and sterilisation may also have an impact. The sterilization program of product temperature of 121.1C for 2hrs12min was calculated based on the above parameters and was found sufficient for sterilizing the pates to make them microbiologically safe for consumption. The price of tin cans and jars being approximately the same, glass jars were considered as a better choice as it is more attractive to the consumer (since the product can be seen).

### 3.3. Microbiological analysis

After 2 weeks of packaging and sterilization, the pate samples were analysed for microorganisms responsible for spoilage of packaged foods. Sulphite-reducing clostridia, lactobacillus nor coliforms were detected in 25g of pate. The sample also tested negative for yeasts and moulds. This confirms that the sterilization process is adequate to make the food microbiologically safe to consume. Smoking and canning are, historically, the best methods to preserve fish and fish products and increase its shelf-life (Ghaly et al., 2010).

### 3.4. Nutritional analysis

Knowing the content of protein, fat, and carbohydrates in the components of the product, their energy value and percentage in the recipe, nutritional value of the final product (sample 1) was calculated. Other food additives (flavours and blends) did not have food or energy value and hence their contribution to the proximate values was ignored.

Energy value was calculated as follows (From Table 1 and Table 2):
E = 11.32*9+45.3*1.37+11.32*0.41 = 101.88 + 62.061 + 4.5 = 168.45 kcal/100 g of product

Protein, fat and carbohydrates were calculated as follows (From Table 1 and Table 2):
Protein = 0.453*12.1 + 0.11*1.4 = 5.48 + 0.154 = 5.634 g/100g of product
Fat = 0.453*12.6 + 0.11*9 = 5.7+0.99 = 6.69 g/100g of product
Carbohydrates = 0.7*3.4 + 0.82*11.32 = 2.38 + 0.902 = 3.3 g/100g of product

The nutritional value of the smoked sprat heads pate (per 100 g) was: Proteins – 5.634 g, fats – 6.69 g, carbohydrates – 3.3 g (including ballast carbohydrates – 2.38 g) and

energy value – 168.45 kcal/100 g. Ballast substances are indigestible carbohydrates, which have zero caloric value, but play a huge role in proper digestion. In the present formulations, wheat fibre is used as ballast substances.

### 3.5. Organoleptic analysis

Sensory analysis is vital not only for developing new products but also for process optimisation and improvement (Sidel & Stone, 1993). Fig. 1 shows the organoleptic evaluation results of the 4 pate samples (composition given in Table 1). In the sensory evaluation participated nineteen independent evaluators, fully trained before carrying out the tasting. It can be observed that all the pate samples have scored between "average" to "good" during the evaluation process for all the attributes: colour, aroma, texture, taste, aftertaste, and appearance. Overall, samples 1 and 2 were better compared to samples 3 and 4 in almost all respects. Sample 1 was chosen for subsequent studies over sample 2 as it scored higher with respect to very important attributes like appearance, texture, taste and aftertaste. Although the scores other attributes such as colour and aroma were slightly better for sample 2, it suffered a serious drawback of have more number of ingredients, especially the fillers. The higher fish head content and presence of vinegar in Sample 1 may be responsible for imparting better taste and texture to the final product.

The result of the MANOVA indicated a significant difference, Wilk's Lambda = .51, $F(15,188) = 3.42$, $p < .05$, $\eta 2 = .20$. Follow up comparisons indicated that opinion difference across all organoleptic factors was significant, $p < .05$, except Texture, $p > .05$, suggesting that texture got similar evaluation across all pate samples. Further paired comparisons (using LSD correction) as shown in Table 3, revealed that colour, aroma and taste of Sample 1 and Sample 2 were scored higher than Sample 3 and Sample 4. Aftertaste of Sample 1 was better that of Sample 2, therefore Sample 1 has been chosen for further experiments.

### 3.6. Flavoured pates

Future studies are aimed at product improvement in terms of making the product cheaper by using cheaper ingredients. For example, by replacing the TARI blends by considerably cheaper mono-ingredient blends. On the basis of the neutrally flavoured pate, it is possible to produce a wide range of products with more complicated flavours. In terms of the organoleptic criteria, the below pates have been qualified and are being currently tested:

- "Borodinsky" – with cumin and cardamom, and "Caramel – burnt sugar" colouring;
- "Chilly" – with red hot chilli pepper flakes and grains. This pate is coloured using the liquid paprika extract;
- "Mediterranean Herbs" – with dried rosemary and thyme;
- "Tomato" pate containing 80 % of pate and 20 % of tomato paste;
- "Classical" – it is the base pate coloured using the "Caramel – burnt sugar" colour.

**Table 1.** Formulations of four pate samples that yielded the best results based on taste and texture

| Ingredients | % (w/w) | | | |
|---|---|---|---|---|
| | **Pate Sample 1** | **Pate Sample 2** | **Pate Sample 3** | **Pate Sample 4** |
| Water | 19.7 | 30.6 | 50 | 0 |
| Vegetable oil | 11.3 | 11.2 | 0 | 29.6 |
| Tari Combi Pate | 3 | 2.5 | 8 | 0 |
| Salt | 1.4 | 1 | 1.1 | 0 |
| Fish heads | 45.3 | 20.3 | 33.7 | 50 |
| Apple cider vinegar | 3.4 | 0 | 0 | 0 |
| Soy isolate | 0 | 4 | 7.2 | 14.3 |
| Wheat fiber (filler) | 3.4 | 3.5 | 0 | 0 |
| Pea flour (filler) | 0 | 2.7 | 0 | 0 |
| Semolina (filler) | 0 | 12.8 | 0 | 0 |
| TARI P 22 | 0.9 | 0.9 | 0 | 0 |
| Black pepper | 0.3 | 1 | 0 | 0 |
| Onion | 11.3 | 9.5 | 0 | 6.1 |
| **Total** | **100** | **100** | **100** | **100** |

**Table 2.** Ingredient list (which contribute to food value) and their composition in pate sample 1

| **Ingredient** | **Protein %** | **Fat %** | **Carbohydrates %** | **Ballast carbohydrates %** | **Energy value kcal/g** |
|---|---|---|---|---|---|
| Sprat (head) | 12.1 | 12.6 | 0 | 0 | 1.37 |
| Wheat fibre (Cellulose) | 0 | 0 | 70 | 70 | 0 |
| Onion | 1.4 | 0 | 8.2 | 0 | 0.41 |
| Vegetable oil | 0 | 100 | 0 | 0 | 9 |

**Table 3.** Results of MANOVA analysis comparing different pate samples and organoleptic parameters

| **Colour** | **Aroma** | **Taste** | **Aftertaste** |
|---|---|---|---|
| #1 better than #3<br>#2 better than #3 #4<br>#1=#2 #4<br>#2= #1 | #1 better than #3 #4<br>#2 better than #3<br>#1=#2<br>#2=#1 #4 | #1 better than #3 #4<br>#2 better than #3#4<br>#1=#2 | #1 better than #2 #3<br>#2 = #3 #4 |

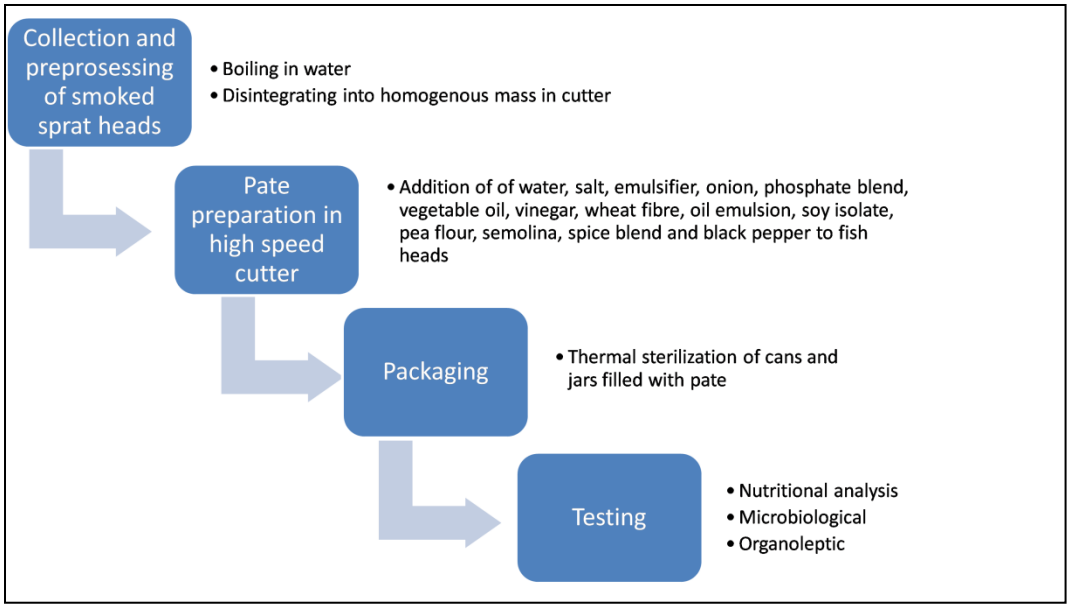


**Figure 1.** Process chart for preparation of fish head pate

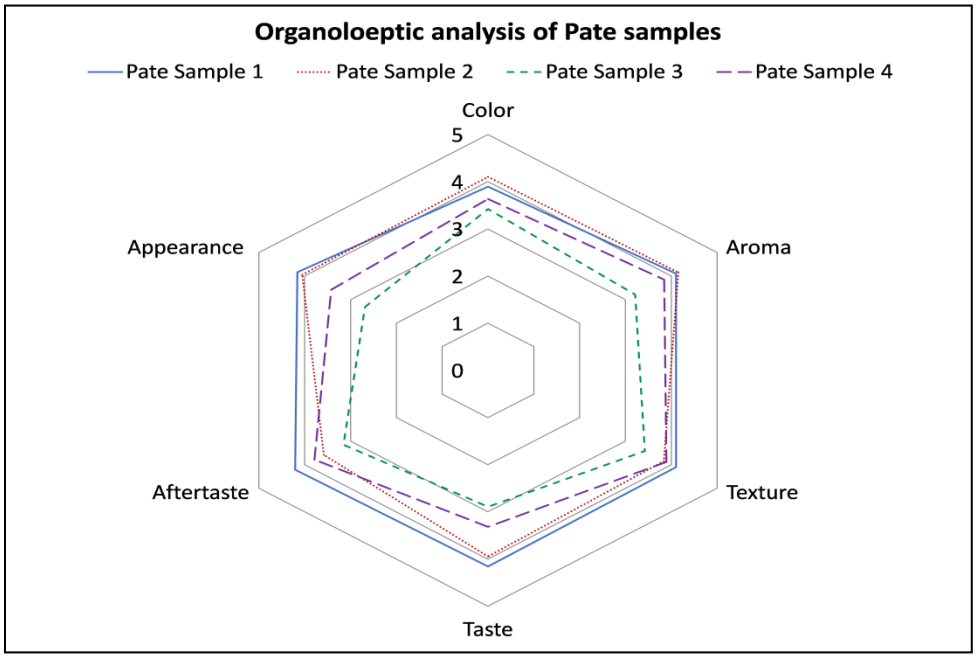


**Figure 2.** Organoleptic analysis of smoked Sprat heads pates

## 4. Conclusions

This study indicated a viable possibility to produce an edible pate out of heads of smoked sprats with minimal processing and low-cost ingredients. A simple high-speed cutter was used in conjunction with the emulsifying agent for the preparation of pate rather than using expensive sophisticated machinery. A neutral flavoured pate with good organoleptic properties has been successfully produced. Flavoured fish pates such as Borodinsky, Chili, Mediterranean herbs, Tomato and Classical flavours are being evaluated. This process has the

potential to convert the underutilized smoked fish waste into edible product, providing an environmental as well as economic solution to fish processing companies who are looking to expand their product range.

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# QUANTITATIVE ANALYSIS OF PROANTHOCYANIDINS (TANNINS) FROM GRAPE (*VITIS VINIFERA*) SEEDS BY REVERSE PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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**ABSTRACT**

Proanthocyanidins(PAs) are oligomeric and polymeric end products of the flavonoid biosynthetic pathway. They are present in the fruits, bark, leaves and seeds of many plants, where they provide protection against predation. At the same time they give flavor and astringency to beverages such as wine, fruit juices and teas, and are increasingly recognized as having a health promoting on human health. Seed extracts from five grape cultivars (*Vitis vinifera*) growing in El-Tarf (Algeria), were screened for their PAs composition and mDP (mean degree of polymerization). The study was realized by means of reversed-phase high-performance liquid chromatography coupled with photodiode array detector (RP-HPLC-DAD) analysis after thiolysis. The study revealed the presence of seven phenolic compounds belonging to the class of flavan-3-ol; Qualitative and quantitative differences among the cultivars were observed. The results confirm that grape seed of varieties studied are a potential source of PAs and can be used as easily accessible source of natural antioxidants.

## 1.Introduction

The polyphenolic compounds known as condensed tannins, or proanthocyanidins (PAs), are plant secondary metabolites synthesized via the flavonoid biosynthetic pathway. They occur in a wide range of plants and play an important role in defense against herbivores. PAs act as powerful antioxidants with beneficial effects for human health including protection against free radical-mediated injury and cardiovascular disease and exhibit a strong antitumor and antimicrobial activity (Veluri *et al*., 2006 ; Mink *et al.*, 2007; Doss *et al*., 2009), PAs also contribute to the astringency and taste of many fruits and other plant products, such as fruit juices, tea and wine. It is well known that the concentration of polyphenolic compounds in grapes depends on the grape cultivar (Mattivi *et al.*, 2006) and other factors, such as ripening time, climate, soil and location of growth (Kennedy *et al.*, 2000). Several methods for the analysis of PAs have been proposed in the literature. Most of them are based on high performance liquid chromatography (HPLC) coupled with a photodiode array (PDA) detector. The aim of this study was to determine the PAs composition of grape seed extracts from

*Vitis vinifera* L. cv., Gros noir, Muscat noir, Cardinal, Muscat blanc and Victoria, all grown in the same geographical area and vintage. Two grape cultivars, Muscat blanc and Victoria, have a yellow- -green colored grape berry. Gros Noir and Muscat noir are navy-blue colored grape cultivars and Cardinal is purple-colored grape cultivar. These entire table grape varieties studied are mostly widespread in this region of Algeria. These grapes can be used fresh as well as for juice production. A RP-HPLC-DAD-UV-Vis method was used for the PAs analysis. The similarities and differences between the PAs compositions of grape seed extracts from different cultivars are discussed.

## 2. Material and methods

### 2.1.Plant material

Seeds from five grape cultivars, including Gros noir, Muscat noir, Cardinal, Muscat blanc and Victoria were examined. Samples collected at maturity, grown in the region of El-Tarf located in North-East of Algeria (36° 45' 00" N; 81° 10' 00" E).The experimental vineyard was raised in 1980 (cultivars Gros noir and Cardinal), 1973 (cultivars Muscat noir and Muscat blanc); The distance of sowing was 3 ×1 m, with two rows support, and the training system was a "double-branched asymmetrical cordone" and 2011 (Victoria) with a distance of sowing of 3 x 3m, with two rows support, and the training system was a Pergola. Approximately 2kg of grape were collected for each cultivar in late summer 2012, from three different sites. All the samples were collected when the Brix values were in range 17 –21°Brix.

### 2.2. Sample preparation

Seeds from berries were manually separated from pulp, then, dried in oven at 50°C until constant mass, then, they were ground to a powder in a domestic mill, stored a -18°C until analysis.

### 2.3.Extraction of flavonoïds from grape seeds

Dried seed powder (0.1g) is subjected to extraction by maceration in 50 ml of a mixture of acetone/water (60:40) and 300µl of methyl-4-hydrobenzoate (1g/l), with stirring for 70 min, the extract was centrifuged (10°C/10 min/10 000 rpm). The supernatant was then filtered through glass microfiber filter GF / A 1.6µm. The solvent was removed to about dryness under reduced pressure by use of a rotary evaporator Buchi® R-111 at 30°C and redissolved in methyl alcohol to a volume of 5 ml to get a crude seed polyphenolic extract.

### 2.4. Isolation of PAs

To separate PAs from crude seed extracts, 2 ml of these extracts were subjected to chromatography over Fractogel Toyopearl® HW-40(F) (300 mm × 10 mm i.d.) (Tosoh Corporation, Japon). Anthocyanins, flavonols, monomeric and dimeric flavanols were eliminated with 30 ml of ethyl alcohol/water/TFA (110:90:0:01) as the eluant at 1ml $min^{-1}$ and PAs fraction was eluted with 30 ml of acetone/water (60:40) at 1ml $min^{-1}$. To this fraction, 300 µl of internal standard (50 mg of methyl 4-hydroxybenzoate in 100 ml of Methanol) was added. The acetonic fraction was dried using a rotary evaporator Bucchi® under vacuum at 30°C and then dissolved in 5 ml methanol for the thiolysis reaction.

### 2.5. Characterization of PAs

Acetonic fraction was subjected to thiolysis reaction, this was performed as described previously (Cadot *et al*., 2012) in duplicate, after addition of 120µl of toluene-α-thiol to 120µl to the fraction above and heating for 2 min at 90°C, this reaction allow the distinction between terminals units (released as flavan-3-ols) and extension units (released as the corresponding benzylthioether derivatives). Ration between

total unists and terminal units gives access to the mDP.

The thiolysis reaction medium (20 µl) filtrated through a membrane filter with an aperture size of 0.45 µm was analyzed by RP-HPLC.

Identification and quantification of PAs was carried out using analytical reversed-phase HPLC according to the conditions adapted from those described by Brossaud et al. (1999), in a Waters Millenium HPLC–DAD system (Milford, MA) system with an auto-sampler and quaternary pump coupled to a diode array detector. A 250×4.6 mm (internal diameter), 5 µm, reversed-phase Lichrospher 18 RP100, column (Merck, Darmstadt, Germany) was used and the elution solvents were: A, water/acetic acid (97.5:2.5) and B, acetonitrile /water / acetic acid (80:17.5: 2.5); isocratic elution with 100% A for 5 min, followed by linear gradients from 100% to 90% in 30 min; from 90% to 80% in 30 min, from 80 to 0% in 5 min, from 0% to 100% in 5 min, washing and re-equilibration of the column. The column temperature was at 30°C, the flow-rate was set at 1 $ml.min^{-1}$ and detection was monitored at 280nm. Peak identification was performed by comparison of retention times and UV –Vis spectra. Each sample of berries was extracted in duplicate and the acetonic fractions were analysed in duplicate too. Hence, the final result was the arithmetic average of four analyses.

### 2.6.Statistical analysis

Results expressed as mean ± standard deviation (SD). Statistical analysis was carried out using the STATISTICA software version 5.0 (Copyright© StatSoft, France). Differences between means were first analyzed using the ANOVA test and the least significant differences (Fisher's LSD) were calculated following significant *F* test ($P \leq 0.05$).

## 3.Results anddiscussions

### 3.1. General

Condensed tannins or PAs are characterized by the properties to give combinations with proteins and other polymers such as polysaccharides. The tannins are characterized by a sensation of astringency (dry mouth).

The cultivars of *Vitis vinifera* selected for this study are to date widely cultivated in this area. Muscat noir, Cardinal and Muscat blanc are the main Algerian varieties, followed by Gros noir, which has a limited cultivation area. Victoria is an Italian variety, recently introduced in Algeria.

Analysis of polyphenols was carried out on the grape seeds, because this is part of berry that contains main class of polyphenols PAs (Guerrero *et al.*, 2009).

### 3.2. PAs and derivatives

Depolymerization in the presence of acid and nucleophile followed by HPLC analysis is a useful tool for quantification and characterization of PAs. This method allows determining the nature and concentration of terminal and extension units and consequently calculating the mDP and the percentage of galloylation (%ECG) of PAs using toluene--α-thiol (benzyl mercaptan).

The PAs are listed in Table 1 (20 – 26). The total amount of PAs (TP, see Table 2), ranging from 565.70±113.56 mg/g (Victoria) to 1080.35±87.04 mg/g (Gros noir) which is not statistically different from the Muscat noir.

**Table 1**. Retention time of different PAs compounds in different table grape varieties

| Compound | | Retention time (min) |
|---|---|---|
| **Proanthocyanidins** | | |
| **20** | Catéchine (C) | 8.100 ± 0.079 |
| **21** | Epicatéchine (EC) | 9.112 ± 0.075 |
| **22** | Epicatéchine-3-O-Gallate (ECG) | 11.609 ± 0.129 |
| **EI** | Etalon interne (EI) | 15.006 ± 0.127 |
| **23** | Epigallocatechine-SH (EGC-SH) | 18. 263 ± 0.129 |
| **24** | Catechine-SH (C-SH) | 20. 822 ± 0.136 |
| **25** | Epicatechine-SH (EC-SH) | 21.563 ± 0.134 |
| **26** | Epicatéchine-3-O-Gallate-SH (ECG-SH) | 23.725 ± 0.128 |

**Table 2.** Levels of TPAs, mDP, % CGE and % ECG of seed of different grape varieties analyzed

| Variety | TP (mg/g of berries) | DPm | % ECG | % EGC |
|---|---|---|---|---|
| **Muscat B** | 1935.85±60.88$^{d}$ | 5.68±0.02$^{d}$ | 26,25 ± 0.004$^{d}$ | 0.00 |
| **Gros Noir** | 1080.35±87.04$^{c}$ | 4.77±0.10$^{c}$ | 23,45±0.001$^{c}$ | 0.00 |
| **Cardinal** | 871.95±45.04$^{b}$ | 4.50±0.01$^{b}$ | 21.52±0.003$^{a}$ | 0.00 |
| **Muscat N** | 932.75±10.96$^{b,c}$ | 4.52±0.07$^{b}$ | 21,28±0.012$^{a}$ | 0.00 |
| **Victoria** | 565.70±113.56$^{a}$ | 4.14±0.14$^{a}$ | 22,00±0.001$^{a,b}$ | 0.00 |

*TPAs: Total Proanthocyanidins*
*DPm: Mean of Polymerization Degree*
*ECG: Epicatechinegallat*
*EGC: Epigallocatechin*
*Results expressed as mg per g of berries. Values with the same letter in each column do not differ significantly ($p < 0.05$). The results are classified in ascending order; a <b <c <d.*

Based on works of Brossaud et al. (1999) on Cabernet franc berries grown on different sites of the vallée de la Loire (France) - 1995 vintage, the content of seed PAs (condensed tannins) oscillate between 3.363 and and 4.448 g / kg fresh weight. On other study, Lorrain et al. (2011) obtained on two French red grape varieties Cabernet sauvignon and Merlot, PAs seed contents ranging from 90.1 ± 4.0 and 92.2 ± 4.5 mg/g of dry weight, respectively.

The levels of grape PAs vary considerably, depending on the variety, environmental conditions, especially water supply and sunlight exposure, berry size and number of seeds (Cadot, 2010), harvest year (Sun *et al.*, 2001), the degree of maturation (Jordão *et al.*, 2001; Ó Marques *et al.*, 2005); these differences also highlight the impact of different soils, cultural practices, but also the harvest in metabolism way of tannins (Lorrain *et al.*, 2011). According to Mateus et al. (2001), low altitudes appear to be favorable for the synthesis of high concentrations of PAs in relation to weather conditions.

Muscat blanc variety showed the highest flavanol content (1935.85±60.88 mg/g of berries). These findings are consistent with previous reports relating to grape varieties grown around the world (Negro *et al.*, 2003; Rockenbach *et al.*, 2011) and confirm that

grape seed extracts are a rich source of PAs, usually oligomers and polymers of polyhydroxy flavan-3-ols such a (+)-catechin and (−)-epicatechin, in the form of gallate esters or glycosides.

Results of Cadot et al. (2006) on Grape seeds from *Vitis vinifera* L. cv Cabernet franc suggest that evolution of the tannins in the seeds is related to evolution of cell wall, but more investigations should be done, in particular, about the composition and structure of the grape seed cell wall and the evolution of the cellular structures, the oxidation of phenolic compounds and their implication in the changes in cells walls, and the impact of cellular death on the extractability of PAs.

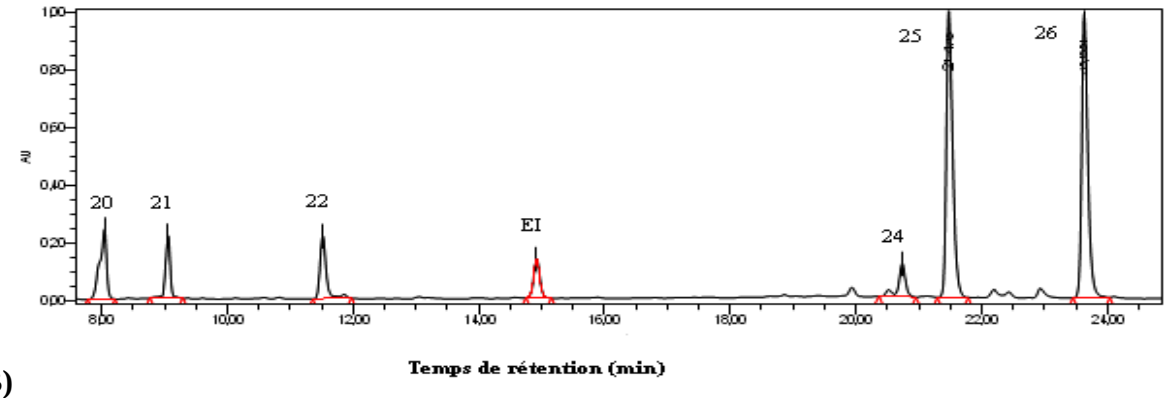
The content of total monomeric and oligomeric flavanols was estimated by thiolysis assay, the monomeric form of PAs represented by the flavan-3-ols epicatechin and catechin monomer were detected in seed extracts, but could not be quantified (peak between thresholds detection and quantification) (Fig. 1 and 2), this is probably due to the fact that catechin is present in a small proportion (less than 10%) (Sun *et al.*, 2001), or to an inter-conversion between catechin and EGC during the maturation of grapes analyzed. According to Liang et al. (2012), the contents of (+) -catechin and epicatechin are significantly lower than those of the other two monomers (ECG and EGC).

mDP and % ECG values recorded vary significantly from one variety to another ($p < 0.05$) from 4.14 ± 0.14 (Victoria) to 5.68 ± 0.02 (Muscat blanc) and 21.28 ± 0.012 (Muscat noir) - which does not differ significantly from Victoria - to 26.25 ± 0.004% (Muscat blanc), respectively. The work of Obreque-Slier et al. (2010) on grape of two varieties (Carmenere and Cabernet Sauvignon) from Chile, show mDP of 2.0 ± 0.2 and 1.8 ± 0.2, while the ECG varies between 20.6 ± 5.5 and 18.7 ± 5.5 (seeds) for the two varieties respectively.

Muscat blanc and Cardinal exhibit a high mDP when comparing with others varieties, which explains their astringency character. According to Cadot et al. (2006), the homogeneous polymerization during the PAs synthesis between fruit set and ripening increases astringency as they increase in size, while the combination with anthocyanins decreases the reactivity, and therefore the astringency of the compounds formed. Regardless of the vintage, the mDP and the percentage of galloylation of seed tannins appear to be appropriate tools to discriminate Muscat blanc and Cardinal variety from others.

The values obtained for the mDP, show that seed tannins are in oligomeric and monomeric forms (mDP varies from 2 to 12-15) (Jordão and Correia, 2012).

**A)**

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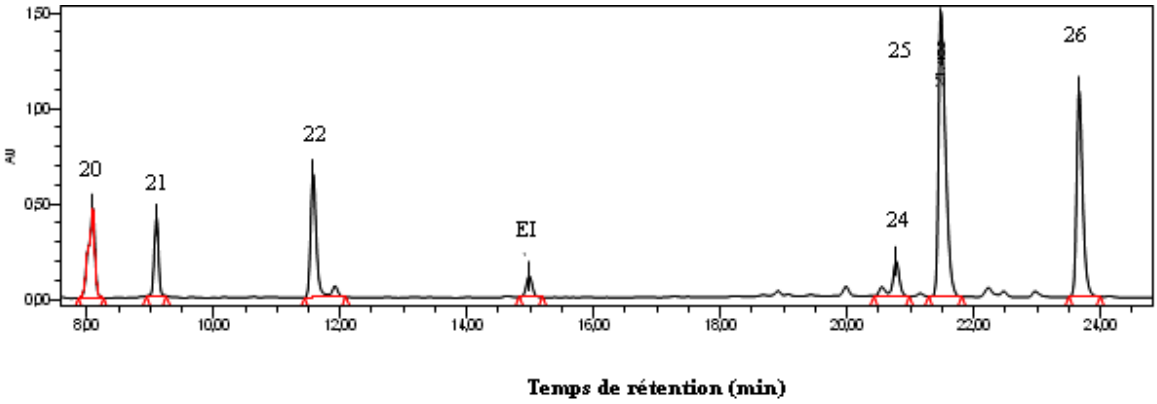


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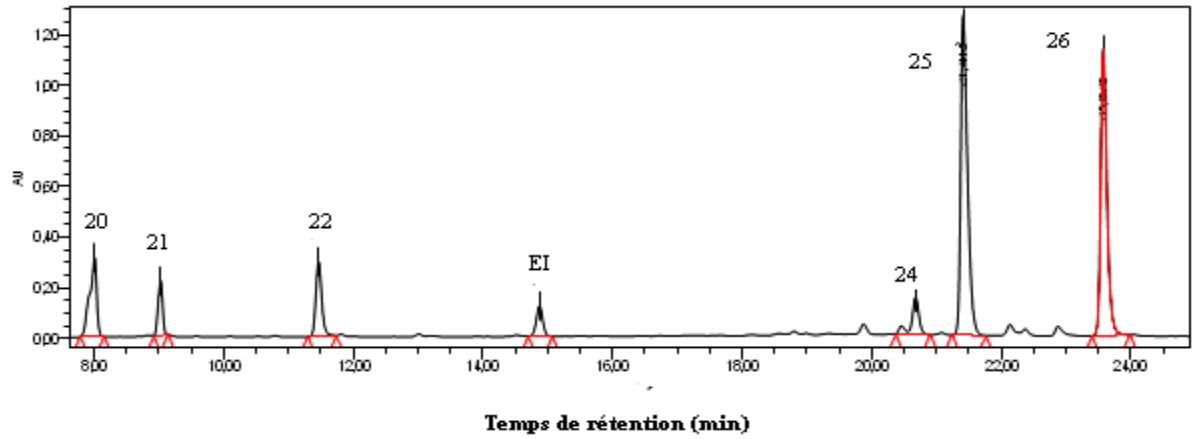
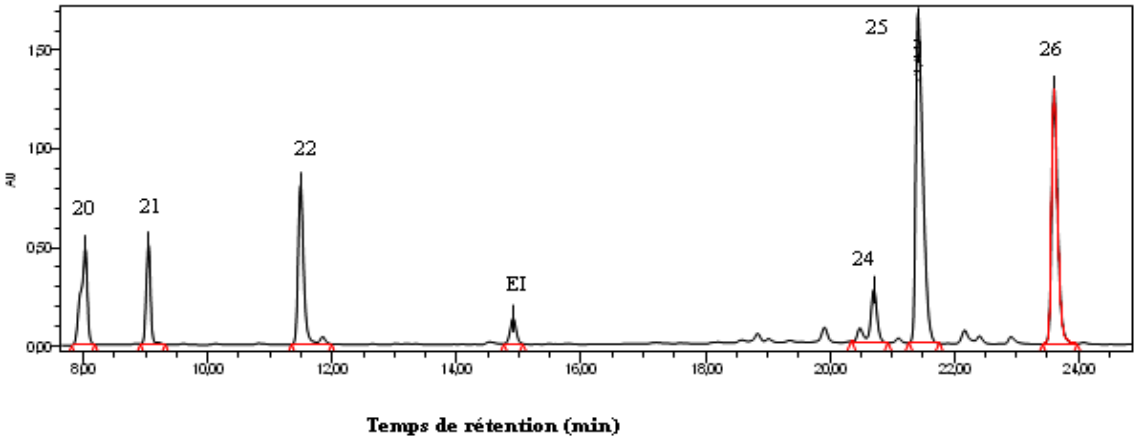



**Figure 1.** Typical PAs HPLC trace of a seed grape extract monitored at 280nm of Gros noir **(A)**, Cardinal **(B)** and Muscat noir **(C)**

**A)**




**B)**

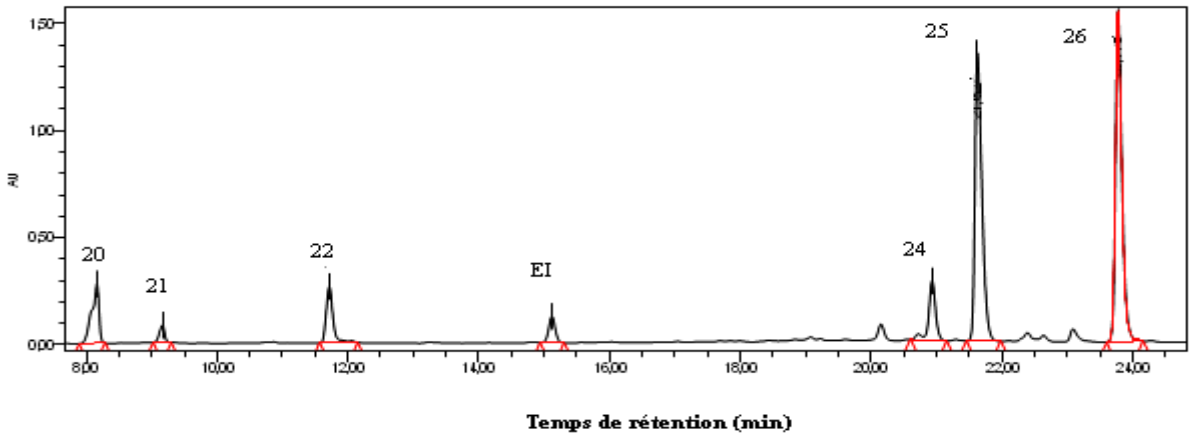



**Figure 2.**Typical PAs HPLC trace of a seed grape extract monitored at 280nmof Victoria **(A)** and Muscat blanc **(B)**

## 4. Conclusions

Results obtained show that the PAs composition and its various subunits differ significantly from one variety to another. This could be due to differences in the astringency of the studied varieties. Given the heterogeneity of these observations, the debate remains open about the possible changes affecting the tannins during the maturation phase. Today it is known with the contribution of new biochemical analysis techniques and knowledge on the expression of genes involved in the production of these compounds as active biosynthesis of tannin of the skin and seeds (the pulp also ) takes place in the green stage, when the formation of the berry. During ripening, there is no accumulation of tannins, wherease the synthesis of anthocyanins takes place, the biosynthetic pathway is partially common to that of tannins. This study show also, that the mDP and the percentage of galloylation of seed tannins can be used as a significant tools to discriminate Muscat blanc and Cardinal variety from others.

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# RELATIONSHIP BETWEEN CASEINATE/CARRAGEENAN EDIBLE FILM AS LACTIC ACID BACTERIA CARRIER AND ITS ANTIMICROBIAL ACTIVITY AGAINST PATHOGENS IN VITRO: EFFECT OF CARRAGEENAN TYPE

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**ABSTRACT**

Edible films can be elaborated with proteins and/or polysaccharides. Composite edible films combine the functionality of each component, enhancing physical properties. In this research edible films were elaborated with sodium caseinate and different types of carrageenan (iota, kappa or lambda), to manipulate the interaction among them as carrier of lactic acid bacteria. Lambda carrageenan resulted in less soluble and tougher edible films, due to their higher sulfate group content and higher interaction with proteins. Higher solubility and hence a less ductile film structure enhanced P. pentosaceus bacteria viability and its antimicrobial activity, at least for Listeria and E. coli. The solubility and structural characteristics of caseinate edible films can be manipulated depending on carrageenan type employed, to enhance their capacity to active packaging for probiotic bacteria.

## 1.Introduction

Edible films are elaborated with any GRAS material employed to enrobing foods to extend shelf-life and that may be eaten together with the food, providing replacement and/or fortification of natural layers to avoid moisture losses and control gases exchange, to prevent loss of important components, with a thickness lower than 0.3 mm (Pavlath and Orts, 2009). The functionality of edible films depends on the nature of the different components and on their final composition and structure (proteins and polysaccharides). In general, polysaccharide based films absorbs more water and are more readily disintegrated with poor barrier properties than protein based films (Cuq et al., 1995). Polysaccharides impart structural cohesion serving as structural matrix, and proteins give rise to a very firm structure by both inter- or intramolecular folding and interaction (Wu et al., 2002).

The polyelectrolyte character of milk proteins in the interaction with hydrocolloids like carrageenan plays an important role in determining mixed biopolymer behavior (Dickinson, 98). Caseins and caseinates can readily form edible films from aqueous solutions since caseins are quite soluble in water due to the structure and amino acid composition of caseins, it is likely that hydrogen bonds, electrostatic interactions

and most probably hydrophobic forces are involved in the formation of casein-based edible films (Schou et al., 2005; Frinault et al., 2006). The number and position of sulfate groups entails a negative charge that affects the functionality of the different carrageenan types (Langendorff et al 2000). Carrageenans, which are film-formers, are used mainly in the food industry as texturizing agents with potential use as coating agents that control transfer of moisture, gases, flavors, and lipids in diverse food systems (Soliva-Fortuny et al., 2012).

The incorporation of antimicrobial agents to packaging materials slows down their release and helps keeping high concentrations of the active compounds on the product surface for extended periods of time (Kristo et al., 2008). Many antimicrobials are proposed to be used in the formulation of edible films and coatings to inhibit the spoilage flora and to decrease the risk of pathogens. There is a trend to select the antimicrobials from natural sources and to use generally recognized as safe (GRAS) compounds to satisfy consumer demands for healthy foods, free of chemical additives (Devlieghere et al. 2004). The advantage in having a film material carrying a biocide is that continued inhibition can occur during storage or distribution of the food product. Application of package-based biocides to reduce post process growth of food pathogens has shown promise, since biocides incorporated into the matrix of films will release their antimicrobial activity to the surrounding environment. To place an antimicrobial hurdle during storage would reduce the chance of cell numbers increasing to a dangerous level (Dawson et al, 2002).

The objective of this work was to evaluate physical and mechanical properties of sodium caseinate edible films with different types of carrageenans (iota, kappa and lambda) to employed as carrier for thermotolerant lactic acid bacteria evaluating their effect on viability and antimicrobial capacity in vitro.

## 2. Materials and methods

### 2.1. Edible films elaboration

Edible films were elaborated with sodium caseinate (DVA Mexicana, Naucalpan) and glycerol as plasticizer and carrageenan. In order to establish the effect of carrageenan type, Viscarin SD389 iota carrageenan, Viscarin GP209 lambda carrageenan, or Gelcarin GP8612 kappa carrageenan (FMC Biopolymers, Philadelphia) were employed. Edible films were prepared according to the casting technique, dehydrating the filmogenic protein-carrageenan-plasticizer solution. Sodium caseinate (8%, w/v) was dissolved in 100 mL of distilled water, adding glycerol (0.4%, w/v) and each carrageenan type (0.3%, w/v). Solutions were poured in glass plates (12×12 cm2) and dehydrated at room temperature (25±1 °C) at 55±5% RH during 48-60 h. Afterward, edible films were kept in desiccators for further analysis.

### 2.2. Total soluble material and soluble protein

Total soluble material was determined according to the method reported by Pereda *et al.* (2012). Edible films samples (2×2 cm) were weight and immersed in 30 mL of water during 24 h. After immersion, samples were oven dried at 105 °C during 24 h to determinate the insoluble material. Total soluble material was reported as the percent of dissolved mass (dry basis) with respect to the initial film dry weight.

From the distilled water employed in total soluble material, soluble protein was determined by biuret method (Gornall *et al.*, 1949). Film soluble protein was reported according to Jangchud and Chinnan (1999):

$$\text{Soluble protein (\%)} = \frac{\text{Protein concentration in 30 mL}}{\text{Initial film weight} \times \text{\% protein in film} \times \text{\% film dry matter}} \quad (1)$$

### 2.3. Mechanical properties: Puncture and tension tests

Force and deformation at the breaking point was determined according to the described by Sobral *et al.* (2001). Samples were fixed in 52.4 mm diameter acrylic cells and perforated in the center with a 3 mm aluminum probe at a constant rate of 1 mm/s in a LFRA 4500 texturometer (Brookfield Engineering Laboratories, Middleboro). From time-force curves, puncture force (maximum force at film breakdown) was reported and puncture deformation was calculated as:

$$\text{Puncture deformation (\%)} = \frac{\Delta l}{l_0} = \frac{\sqrt{(D^2 + l_0^2)} - l_0}{l_0} \times 100 \quad (2)$$

Considering that the stress was perfectly distributed along the film, where D is probe displacement, l0 is the initial film length (radius of the measurement cell, 26.3 mm).

Film tensile strength and elongation percent were determined employing a Chatillon TCM 200 motorized test stand with a DFIS 200 digital force gauge according to the described by Gennadios *et al.* (1993). Films samples were cut in 100×25.4 mm and placed in the grips with an initial separation of 50 mm. Samples were stretched at a constant speed rate of 1 mm/s until breakdown. Tensile strength was calculated dividing the peak load by the cross-sectional area (film width×thickness). The elongation percent was calculated as the ratio of the extension values and the initial grip separation multiplied by 100.

### 2.4. Lactic acid bacteria incorporation into edible film

Lactic acid bacteria *Pedioccocus pentosaceus* UAM22, previously reported as thermotolerant and probiotic (Ramirez-Chavarín et al, 2010; Ramírez-Chavarin et al, 2013), was prepared reactivating cells in 10 mL MRS broth, incubating at 37 °C for 24 h until an optical density close to one (λ = 600 nm), containing approximately 108 CFU/mL. Cells were harvest by centrifugation at 2000×g during 20 min and washed in sterile water. Bacterial cell preparation (1%, w/v) was added to caseinate-carrageenan solution under magnetic stirring for 5 min before casting process.

### 2.5. Cell viability and antimicrobial activity

Viability of the lactic acid bacteria was determining in edible films stored in petri dishes at 25 °C for 7 days. Edible films were then placed in dilution flask with 100 mL of sterile saline solution homogenizing during 5 min. Serial dilutions were performed and poured on MRS plates, incubating at 37 °C during 48 h before colonies counting.

Antibacterial activity of lactic acid bacteria in caseinate-carrageenan edible films was determined by the disc diffusion technique, the Kirby-Bauer method (Valencia *et al.*, 2013). *Listeria innocua*, a non-pathogen strain, was employed instead *Listeria monocytogenes*, due the physiological similarity (Begot *et al.*, 1997). *L. innocua* was reactivated in 3 mL of brain heart infusion (BHI) media and incubated at 37 °C during 12 h before adding 7 mL of BHI broth to incubate at 37 °C during 24 h. Same procedure was followed for *Escherichia coli* and *Staphylococcus aureus*. A 6-mm diameter disc of each edible film treatments was placed in a petri dish with Meuller-Hilton or BHI agar, previously inoculated with 0.2 mL of *L. inocua*, *E. coli* or *S. aureus* strains inoculums (ca. $10^5$-$10^6$ CFU/mL). Petri dishes were incubated at 37 °C during 24 h to determinate the bacterial growth inhibition zone around the discs. A clear

zone after incubation assumes that the edible film and the antimicrobial compound presented inhibition (Maizura *et al.,* 2007). Inhibitory activity of the edible film as lactic acid bacteria carrier was determined as the inhibition halo diameter ($\pi \times$clear zone radio$^2$).

### 2.6. Experimental design and data analysis

The effect of the different carrageenan type on caseinate edible films on films properties, cell viability and antimicrobial activity was analyzed by PROC ANOVA procedure in SAS Statistical Software version 8.0 (SAS Institute, Cary). Significantly ($P<0.05$) difference among means were determined with Duncan's mean test in same software. The strength and direction of the linear relationship between corresponding variables, edible films properties (total soluble matter, soluble protein, puncture and tensile resistance) and film entrapped lactic acid bacteria (cell viability and antimicrobial activity) was determine with Pearson's correlation analysis with the PROC CORR procedure in same software.

## 3.Results and discussions

### 3.1. Total soluble material and soluble protein

Iota carrageenan containing films presented the significantly ($P<0.05$) higher total soluble material values, and the lower ones was observed in the lambda carrageenan samples. In same manner, significantly ($P<0.05$) lower soluble protein was observed in lambda carrageenan samples, with higher values in the samples containing iota carrageenan (Table 1).

**Table 1.** Edible film physicochemical and mechanical properties

| Carrageenan type | Total soluble material (%) | Soluble protein (%) | Puncture Force (N) | Puncture deformation (%) | Strain force (N) | Elongation (%) |
|---|---|---|---|---|---|---|
| **Iota** | 72.27±12.99 a | 15.36±4.82 a | 12.71±6.97 b | 26.31±4.02 b | 0.476±0.131 c | 38.75±4.56 c |
| **Kappa** | 70.25± 8.37 b | 11.88±5.56 b | 10.49±2.24 c | 24.79±3.05 c | 0.548±0.143 b | 69.17±2.53 b |
| **Lambda** | 62.08±12.04 c | 11.40±5.94 c | 14.09±7.54 a | 27.64±2.17 a | 0.620±0.153 a | 74.74±3.75 a |

a, b, c Means with same letter in same row are not significantly ($P>0.05$) different

It seems that the structural differences in sulphate groups' content can explain the results. Lower sulphate groups were related to higher soluble material and higher soluble protein, since more sulphate groups (3 in lambda, 2 in kappa, 1 in iota) were related to strong electrostatic interaction with positively charged regions in caseinate, decreasing dissolubility of edible films. Caseins interactions with carrageenans are stronger when carrageenan is in the helical conformation (Langendroff *et al.*, 2000; Gu *et al.,* 2005). At the experimental conditions employed (i.e., room temperature) the conformation of kappa and iota carrageenans are temperature dependent to undergo from a coil (disordered state) to helix (ordered state) transition in solution (Černíková *et al.,* 2008), whereas lambda carrageenan is a random coil conformation (Corredig *et al.*, 2011), increasing interactions and reducing free soluble material.

### 3.2. Mechanical properties: Puncture and tensile tests

Caseinate edible films formulated with lambda carrageenan presented significantly ($P<0.05$) higher values of puncture force as compared to edible films formulated with kappa or iota carrageenan. Same behavior was observed in puncture deformation, where lambda carrageenan samples presented significantly ($P<0.05$) higher values (Table 1).

The temperature dependence conformation of iota and kappa carrageenan decreased their interaction capacity at the experimental conditions, resulting in lower interactions with proteins during film formation. Lambda carrageenan, with more sulfate groups and higher electric charge density besides the no temperature dependence on it conformation result in stronger attractive interaction with caseins proteins (Langendorff *et al.,* 2000). This interaction between lambda-carrageenans and

sodium caseinate could contribute to increase the protein particle size giving rise to a more open structure, increasing edible film resistant to puncture (Fabra *et al.*, 2008).

Strain force of caseinate edible films was significantly (P<0.05) higher for lambda carrageenan containing samples. The films elongation was significantly (P<0.05) higher as well for lambda carrageenan samples. Less stretchable films were obtained with iota or kappa carrageenan (Table 1).

Incorporation of polysaccharides as carrageenans increased caseinate edible films stretchability (Fabra *et al.*, 2008). Once, the electrostatic charge of each particular carrageenan rules their interaction with proteins during the film formation. The more intensive absorption of carrageenans on caseins resulted in bridges structuring a more compact network (Langendorff *et al.*, 2000; Černíková *et al.*, 2008). Higher sulphate groups content (3 in lambda, 2 in kappa, 1 in iota) were related to strong electrostatic interaction with positively charged regions in proteins. More interaction resulted in a ductile edible film with higher resistance to be distended. Puncture force, besides tension strain, express the maximum stress developed by the film under extension test (Chiralt *et al.*, 2012).

### 3.3. Cell viability and antimicrobial effectiveness

The viability of lactic acid bacteria for the edible films formulated with the different carrageenans was as following: iota ($8\times10^6$ CFU)> kappa ($4\times10^6$ CFU)> lambda ($0.1\times10^6$ CFU). Table 2 show the results for pathogens inhibition for the different edible films elaborated with caseinate and carrageenans. In Mueller-Hilton media, for *L. inocua* the inhibition ratio was significantly (P<0.05) higher for iota carrageenan samples, and the lower radio was observed in lambda carrageenan samples.

**Table 2.** Inhibition halo diameter in the antimicrobial test of edible caseinate films with Iota, Kappa or Lambda.

| Carrageenan type | Mueller-Hilton agar | | | BHI agar | | |
|---|---|---|---|---|---|---|
| | ***L. innocua*** | ***E coli*** | ***S. aureus*** | ***L. innocua*** | ***E coli*** | ***S. aureus*** |
| **Iota** | 1.65±0.11 a | 2.29±0.10 a | 0.00 | 1.54±0.10 a | 1.69±0.12 a | 0.00 |
| **Kappa** | 1.56±0.12 b | 2.12±0.09 b | 0.00 | 1.45±0.11 b | 1.56±0.12 b | 0.00 |
| **Lambda** | 1.41±0.10 c | 2.00±0.05 c | 0.00 | 1.34±0.09 c | 1.47±0.13 c | 0.00 |

a, b, c Means with same letter in same row are not significantly (P>0.05) different

Same tendency was observed for *E. coli* as well, with no inhibition for *S. aureus*. In BHI agar, iota and kappa carrageenan incorporation into caseinate edible films resulted in significantly (P<0.05) higher inhibition halo radius, with the lower one in lambda carrageenan samples. The anti-Listerial effect of lactic acid bacteria has been attributed to several mechanisms, from acidification due to lactic acid production (Bredholt *et al.*, 2001), competence for nutrients (Vermeiren *et al.*, 2006), bacteriocins production (Katla *et al.*, 2002) or even to non-producing bacteriocins strains (Alves *et al.*, 2006). Probably same mechanism can be applied to E. coli, although at the experimental conditions with the employed thermotolerant lactic acid bacteria strains *S. aureus* was not inhibited.

Lower solubility and tougher structure (in lambda carrageenan samples) seems to be related to lower lactic acid bacteria viability and diminution of antimicrobial capacity. Table 3 presents the correlation coefficients, irrespectively of carrageenan type, for the physical and mechanical parameters of edible

films against their capacity to maintain lactic acid bacteria viable and to inhibit pathogens.

**Table 3.** Pearson's correlation coefficients and significance among variables, irrespectively of carrageenan type.

| Variable | Total soluble material (%) | Soluble protein (%) | Puncture force (N) | Puncture deformation (%) | Tensile strength (N) | Elongation (%) | Cell viability ($\times 10^6$ CFU) | Inhibition halo radius (mm) |
|---|---|---|---|---|---|---|---|---|
| **Total soluble material (%)** | 1.0000 | 0.6329 <0.0001** | 0.7002 <0.0001** | 0.6776 <0.0001** | 0.6736 <0.0001** | 0.3107 0.132* | -0.3905 <0.0001** | 0.0676 0.5986• |
| **Soluble protein (%)** | | 1.0000 | 0.8954 <0.0001** | 0.9448 <0.0001** | 0.4537 0.0002** | -0.1735 0.1740• | 0.3486 <0.0001** | 0.3322 0.0078** |
| **Puncture force (N)** | | | 1.0000 | 0.8489 <0.0001** | 0.3780 0.0023** | -0.1707 0.1810• | -0.2985 0.0200* | -0.3689 0.0029** |
| **Puncture deformation (%)** | | | | 1.0000 | 0.6084 <0.0001** | 0.0907 0.4796• | 0.1388 0.0869• | 0.1928 0.1300• |
| **Tensile strength (N)** | | | | | 1.0000 | 0.7665 <0.0001** | -0.5824 <0.0001** | -0.3784 0.0022** |
| **Elongation (%)** | | | | | | 1.0000 | -0.9135 <0.0001** | -0.6726 <0.0001** |
| **Cell viability ($\times 10^6$ CFU)** | | | | | | | 1.000 | 0.5706 <0.0001** |
| **Inhibition halo (mm)** | | | | | | | | 1.0000 |

** highly significant (P<0.01), *significantly (P<0.05), •not significantly (P>0.05)

The correlation among mechanical properties was coherent, since the positive and highly significant (P<0.01) relationship between soluble material and soluble protein. In same manner, edible films solubility (total soluble material and soluble protein) presented a highly significant (P<0.01) correlation with the films resistance to perforation, being higher (high correlation coefficient value) with soluble protein. For stretch out resistance (tensile strength and elongation), there was a highly significant (P<0.01) relation of tensile strength with solubility properties. Total soluble matter was not significantly (P>0.05) with films elongation, but there was an inverse significantly (P<0.01) correlation of soluble protein with the film stretch capacity. Elongation was not significantly (P>0.05) with both puncture parameters, that presented a highly significant (P<0.01) correlation with tensile strength. These results indicate that the interactions during the filmogenic process of sodium caseinate with the different carrageenans had as consequence distinct solubility characteristics, more over of proteins. More soluble films (proteins released) in water were more resistant to puncture and tension. These properties are important to consider since edible films solubility controls the diffusion release of bioactive compounds to food surface (Quirós-Sauceda *et al.*, 2014), and mechanical resistance is important to maintain edible film integrity during process and handling before

applying to foods (Tanada-Palmu and Grosso, 2003).

Regarding to the correlation amid solubility and mechanical properties with lactic acid bacteria in edible films, total soluble material was highly significant ($P<0.01$) correlated inversely with cell viability, whereas soluble protein presented highly significant ($P<0.01$) correlation with both cell viability and a bigger inhibition halo. The more soluble protein in edible films resulted in better survive of the employed strain *P. pentosaceus*, since these had been reported as thermotolerant, this is, these lactic acid bacteria had an enhanced response to stress (survival during and after edible film process). For mechanical properties, only puncture force presented inverse and significantly ($P<0.05$) and highly significant ($P<0.01$) correlation, respectively, with cell viability and inhibition halo. Both tensile parameters presented an inverse highly significant ($P>0.01$) correlation with both cell viability and inhibition halo. This means that the more ductile or tougher edible film structure restrain lactic acid bacteria. Although the incorporation of lactic acid bacteria into biopolymer films can modify barrier or mechanical properties, application of edible films assures the food protection against moisture changes or mechanical damages (Sánchez-González *et al.*, 2014).

Finally, there was a highly significant ($P>0.01$) correlation between cell viability and inhibition radius. The different properties of edible films, as result of the protein-polysaccharide interaction, different type of carrageenan, affected the viability and metabolism of lactic acid bacteria in their antimicrobial activity. Migration or liberation of antimicrobial depends on the electrostatic interactions between the component and the polymer side chains, ionic osmosis, and possible structural changes induced by the presence antimicrobial compound and the edible film environmental factors like temperature and storage time (Cha and Chinnan, 2004; Lin and Zhao, 2007).

## 4. Conclusions

In caseinate edible films the type of carrageenan type had an influence on their solubility and mechanical properties, parameters that are very important to consider in the handling and application of edible films as bioactive carrier. Probiotic thermotolerant lactic acid bacteria are able to survive edible film casting process, being viable to be applied throughout the edible film as bioactive packaging. Lambda carrageenan resulted in less soluble and tougher edible films, due to their higher sulfate group content and higher interaction with proteins. Higher solubility and hence a less ductile film structure enhanced *P. pentosaceus* bacteria viability and its antimicrobial activity *in vitro*, at least for *Listeria* and *E. coli*. The solubility and structural characteristics of caseinate edible films can be manipulated depending on carrageenan type employed, to enhance their capacity to active packaging for probiotic bacteria.

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# EFFECT OF MUNG BEAN AND RICE ON PHYSICO-CHEMICAL, SENSORY AND MICROSTRUCTURAL PROPERTIES OF CEREAL BARS

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**ABSTRACT**

Cereal bars were formulated using dry raw materials (mung bean and rice). The bars were mixed with dry ingredients at different percentages. The physico-chemical, sensory and microstructural properties of cereal bars after mixing were investigated. The consumer acceptability of the cereal bars were carried out using 9-hedonic scale. All the qualities evaluated significantly ($p \leq 0.05$) affect the acceptability and preference of the samples.

## 1. Introduction

In the present era, consumers are getting more aware about health foods with balanced nutrition. This has diverted the attention of food researchers towards the development of high quality food products with health benefits. Cereals are known to possess a vital role in day-to-day life and are being utilized in preparation of cereal bars, instants and energy bars as they serve the necessary ingredients to functional foods (Silva et al., 2014). Cereal bars, the most convenient foods often used as alternatives to meet the demand of low-calorie or protein enriched products for athletes (Norajit et al., 2011). These bars are easy to manufacture and generally obtained by compacting cereal flakes viz. rice, maize, oats, barley, along with glucose syrup/sugar, natural/artificial sweetener, and dried fruits (Appelt et al., 2015). The energy bars are multi-component and can be very complex in its formulation. The ingredients must be combined in a specific manner to ensure that they complement each other and results into good taste, texture and physical properties (Murphy, 1995). Many fruits have been used extensively in preparation of cereal bars which results in increased vitamin and mineral content (Silva et al., 2014).

Al-Shahib et al., (2003) reported that date flesh contains significant amount of carbohydrates, proteins, lipids, vitamins and minerals. Bioactive compounds from date polysaccharides can be used as a functional constituent in drug formulations (Chaira et al., 2009). Polyphenolic compounds from dates with antioxidant properties have been studied for its protective effect against lipid oxidation (Moure et al., 2000); controls cardio vascular disease and possess antimutagenic property (Vayalil, et al., 2002). Among cereals, corn has highest antioxidant potential followed by wheat, oat, and rice (Adom and Liu, 2002). On the other hand, barley contains essential bioactive component such as β-glucan which is known to possess positive health benefits as lowering blood cholesterol (Behall et al., 2004; et al.,

1989) and glycemic index (Braaten et al., 1991). Barley is a good source of tocopherols and tocotrienols known to reduce serum cholesterol through their antioxidant action (Qureshi et al., 1986). Hence, this study was undertaken to investigate the effect of mung bean and rice on the physio-chemical, sensory and microstructural properties of cereal bar.

## 2. Materials and methods

### 2.1. Raw Materials

The raw materials viz. rice flakes, mung beans, barley, glucose syrup, brown sugar, honey, dry fruit (dates), black cumin, and cocoa butter were procured from a local market of Srinagar, J & K, India.

### 2.2. Preparation of mung bean flour

Mung beans were thoroughly cleaned, washed and dried at $50^{o}$ C for 5 hours. The mung beans flour (MBF) was obtained using grinder followed by sieving (60 mesh sieve). The MBF was then packed in high density polyethylene bags for further use.

### 2.3. Preparation of rice based cereal bars

The dry fruits (dates) were diced and mixed with black cumin powder in mixer followed by addition of rice flakes, barley and MBF along with binding agents (honey and glucose syrup). The contents were heated in an oven at $100^{0}$ C for 15 minutes. After heating, the mixture was allowed to cool. The cooled mixture was then subjected to cutting of rectangular shape bars (11cm x 3cm x 1.5 cm).

### 2.4. Proximate analysis of cereal bars

Proximate analysis of cereal bars was done by AOAC methods (2001).

### 2.5. Color measurement

Color of cereal bars was measured by a spectrophotometer (COLOR TECH PCM Model: 3001476) equipped with a D65 illuminant using the CIE l*, a* and b* color scale. The samples were placed in the petri dish covered with black container to avoid the external lighting. The measurements were made in triplicate at two random locations on the surface.

### 2.6. Free Fatty Acids and Water activity ($a_w$)

Free fatty acids and peroxide values were determined by AOCS (1990) and AOCS (1973) respectively. The water activity of the samples was measured using a water activity meter (Pre Aqua water activity Analyzer Lab) under controlled temperature conditions.

### 2.7. Sensory properties of cereal bars

Hedonic sensory evaluation of different cereal bars was carried out by semi-trained panelists consisting of 7 members drawn from scholars and staff members of Department of Food Technology, Islamic University of Science and Technology Awantipora Kashmir. The analysis was done on the basis of color, taste, texture, appearance on a nine-point hedonic scale (Meilgaard et al., 2006). Attributes were scored on a scale varying from "9 = like extremely" to "1 = dislike extremely". Differently coded samples were presented to panel members one at a time and they were asked to rate their hedonic response on the scale. At the end of this phase, marking of individual products were calculated and the best acceptable product was determined.

### 2.8. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) uses a focused electron probe to extract structural and chemical information point-by-point from a region of interest in the sample. The high spatial resolution of an SEM makes it a powerful tool to characterize a wide range of specimens at the nanometer to micrometer length scales. The cereal bars for SEM were dried overnight till a vacuum oven at 65°C to reduce moisture content. They were cut using a razor blade. Two specimens were taken from each sample, with one cut along the longitudinal axis and the other along the cross section. Each specimen was fixed on the aluminum stub with a copper tape, coated with gold-palladium by an SEM sputter coater (Model E5100, Polaron Instruments Inc., Cambridge MA, U.S.A.). A voltage of 2.5 kV

and a current of 20 mA were applied for 2 min to deposit a conductive layer of 300 A in thicknesses over the specimen. The specimen was then examined with HITACHI Scanning Electron Microscope (Model S-3000H, Japan) at 5kV. Photographs were taken using Polaroid 55 P/N film.

### 2.9. Statistical analysis:

Data were analyzed by one way analysis of variance followed by Duncan's multiple range tests. All data were presented as means ± SE. Significant differences were accepted when the P value was less than 0.05.

## 3. Results & Discussion

### 3.1. Chemical composition of rice flakes, mung bean flour and barley flour

The chemical composition of rice flakes, mung flour and barley flour is presented in Table 1. The results indicated that rice flakes contained highest amount of carbohydrates (77.7%). The MBF had highest ash (3.79%) protein (24.7%) and crude fiber (7.9%). Similar results were reported by Naivikul et al., (1978). It is also evident from the findings that barley flour contains highest fat (2.45%) and significant amount of crude fiber (5.70%) and ash (2.1%). Similar results were observed by Helm et al., (2004) in chemical characterization of dehulled barley.

**Table 1.**Proximate composition of rice flakes, mung bean and barley flour

| Parameter | Rice Flakes | Mung Powder | Barley Flour |
|---|---|---|---|
| Moisture (%) | 15.6 ± 0.02 $^{a, B}$ | 8.60 ± 0.02 $^{c, C}$ | 10.5 ± 0.01 $^{b, B}$ |
| Ash (%) | 1.0 ± 0.04 $^{c, D}$ | 3.79 ± 0.04 $^{a, D}$ | 2.10 ±0.05 $^{b, D}$ |
| Protein (%) | 5.9 ± 0.02 $^{c, C}$ | 24.7 ± 0.02 $^{a, B}$ | 9.1 ± 0.01 $^{b, B}$ |
| Crude Fat (%) | 0.60 ± 0.01 $^{c, D}$ | 2.13 ± 0.01 $^{b, D}$ | 2.45 ± 0.01 $^{a, D}$ |
| Crude Fiber (%) | 0.60 ± 0.01 $^{c, D}$ | 7.9 ± 0.01 $^{a, C}$ | 5.70 ± 0.02 $^{b, C}$ |
| Carbohydrates (%) | 77.7 ± 0.04 $^{a, A}$ | 52.88 ± 0.04 $^{c, A}$ | 70.15 ± 0.05 $^{b, A}$ |

Values are expressed as mean ± standard deviation. . Means having different letters within the same column differ significantly at $p < 0.05$.

### 3.2. Preparation of cereal bars

The selected fruits and cereals were used for preparation of cereal bars are shown in Table 2. The rice based cereal bars were formulated using the rice flakes and MBF (50:0:-$T_0$, 48:2- $T_1$, 46:4- $T_2$, 44:6- $T_3$, 42:8- $T_4$,) in specific proportion. The samples were then analyzed for physico-chemical, color and sensory properties.

**Table 2.** Formulation of cereal bars

| Treatments (%) | Rice (%) | Mung (% ) | Dates (%) | Barley (%) | Black Cumin (%) | Honey (%) | Glucose Syrup (%) |
|---|---|---|---|---|---|---|---|
| $T_0$ | 50 | 0 | 3 | 3 | 0.5 | 25 | 18.5 |
| $T_1$ | 48 | 2 | 3 | 3 | 0.5 | 25 | 18.5 |
| $T_2$ | 46 | 4 | 3 | 3 | 0.5 | 25 | 18.5 |
| $T_3$ | 44 | 6 | 3 | 3 | 0.5 | 25 | 18.5 |
| $T_4$ | 42 | 8 | 3 | 3 | 0.5 | 25 | 18.5 |

Values are expressed on dry weight basis.

### 3.3. Effect of Mung flour on proximate composition of Rice based cereal bars

The effect of mung flour on proximate composition of rice based cereal bars is shown in Table 3. It was observed that moisture content increased significantly from 8.98 ($T_0$) to 14.7% ($T_4$) with increasing level of mung powder. This increase in moisture may be due to fiber present in mung beans powder. These results are in close agreement with the findings of Agbaje et al., (2014) in apricot-date bars and Ahmad et al., (2005) in papaya fruit bars. The cereal bar containing highest enrichment level possess high ash content (3.08%) while as control sample showed lowest ash content (1.70%). This might be due to the mineral content present in mung powder and barley. Similar results were reported in cereals by Cecchi et al., (2003). It was observed that total carbohydrate content of cereal bars showed a decreasing trend (62.07 – 22.45 %), with increasing level of mung powder. The control sample was observed with highest carbohydrate while as cereal bars with highest incorporation level were found with lowest carbohydrates. The reason for decrease in carbohydrate content in cereal bars might be due to low carbohydrate content of mung powder. Carvalho et al., (2011) depicted the similar results for cereal bars made from almonds of chichai, sapucaia and gurgueia nuts. The total protein content of cereal bars showed an increasing trend from 10.37 to 30.47% with increasing level of mung powder.

**Table 3.** Effect of Mung bean flour on proximate composition of Rice based cereal bars

| Parameter | $T_0$ (0%) | $T_1$ (2%) | $T_2$ (4%) | $T_3$ (6%) | $T_4$ (8%) |
|---|---|---|---|---|---|
| Moisture | 8.98 ± 0.02 $^{d, D}$ | 9.9 ± 0.21 $^{d, D}$ | 11.6 ± 0.10 $^{d, C}$ | 13.2 ± 0.20 $^{d, B}$ | 14.7 ±0.18 $^{d, A}$ |
| Ash | 1.7 ± 0.04 $^{f, D}$ | 1.90 ± 0.07 $^{f, C}$ | 2.1 ± 0.08 $^{f, B}$ | 2.21 ± 0.11 $^{f, B}$ | 3.08 ± 0.14 $^{f, A}$ |
| Protein | 10.37 ±0.11 $^{c, E}$ | 12.13 ±0.10 $^{c, D}$ | 22.4 ± 0.12 $^{b, C}$ | 26.31 ± 0.16 $^{b, B}$ | 30.47 ± 0.24 $^{a, A}$ |
| Fat | 12.39±0.08 $^{b, D}$ | 13.05 ± 0.05 $^{b, C}$ | 14.79 ± 0.14 $^{c, B}$ | 15.10 ±0.09 $^{c, A}$ | 15.78 ± 0.18 $^{c, A}$ |
| Fiber | 3.86 ± 0.05 $^{e, E}$ | 5.07 ± 0.04 $^{e, D}$ | 6.85 ± 0.03 $^{e, C}$ | 8.59 ± 0.02 $^{e, B}$ | 12.08 ± 0.01 $^{e, A}$ |
| Carbohydrate | 62.07 ± 0.06 $^{a, A}$ | 57.35 ± 0.05 $^{a, B}$ | 42.27 ± 0.02 $^{a, C}$ | 34.35 ± 0.03 $^{a, D}$ | 22.45 ± 0.02 $^{b, E}$ |

Values are expressed as mean ± standard deviation. . Means having different letters within the same column differ significantly at $p < 0.05$.

The lowest protein content was observed in control sample (10.37%) while as the protein content increased with the addition of Mung powder (30.47%) in $T_4$. The increase in protein content might be due to the appreciable amount of protein present in mung powder. Similar variations in protein content was reported by Lobato et al., (2012) in snack bars with high soy protein and isoflavone content. The fat content of cereal bars ranged from 12.39 to 15.78% significantly. The lowest fat content was observed in control sample (12.39%) and the highest fat content was observed in $T_4$ sample.

### 3.4. Physicochemical analysis of Rice based Cereal bars

The different parameters like water activity, fatty acids, peroxide value and browning index of the rice based cereal bars are shown in Table 4.

#### *3.4.1. Water activity*

The water activity of cereal bars varied from each other. The results indicated variation in water activity within different treatments. The lowest water activity was recorded in $T_4$ sample with 8% incorporation level of mung powder. While as highest water activity was found in control sample. This reduction in water activity in cereal bars might be due to lower moisture in mung powder. Estevez et al., (1995), reported

similar results for water activity in cereal nut bars.

### *3.4.2. Fatty acids*

It is evident from the table that the total fatty acid content of cereal bars showed an increasing trend with increase in concentration of mung powder. The highest fatty acid value of 1.11% was observed in samples containing 8% incorporation level of mung powder and lowest value of 1.05% was found in control sample. The increase in fatty acid value might be due to the amount of fatty acid content present in mung powder. Similar results were reported by Padmashree et al., (2012).

### *3.4.3. Peroxide value*

It was evident from the table that the peroxide value in cereal bars decreases with increasing level of mung powder. The highest peroxide value (5.92 meq $O_2$/kg oil) was noticed in control sample while as the lowest value (5.60 meq $O_2$/kg oil) was found in cereal bars having highest level of incorporation. This might be destruction of antioxidants by the steaming of the rice flakes. Similar results have been reported by Shaheen et al., (1995). The results are also in accordance with Padmashree et al., (2013).

### *3.4.4. Browning index*

Browning index of cereal bars showed an increasing trend with highest browning index (0.14 OD) found in $T_4$ sample and lowest browning index (0.07 OD) was found in control sample. The results are in agreement with the findings of Padmashree et al., (2013) for protein rich composite cereal bars.

### *3.4.5 Color values of cereal bars*

Color analysis of the cereal bars shown in Table 5, depicted that the lightness, redness and yellowness values decreases as the concentration of mung, barley and rice flakes increases. Control sample got the highest value for lightness (l*), redness (a*) and yellowness (b*) respectively which indicated that there is little effect of caramelization and Millard browning in cereal bars. The results are in close agreement with the findings of Murillo et al. (2011).

## 3.5. Effect of Mung powder on sensory perception of rice based cereal bars

Five parameters viz., appearance, color, taste, texture and overall acceptability were used to analyze the best developed optimized product. The results shown in Table 6 represent the lowest and highest color score for $T_4$ (5.62) and $T_0$ (6.64) respectively. This color darkening of cereal bars might be due to sugar caramelization and the Millard reaction between sugars and amino acids. The results are in consonance to the findings of Barros et al., (2011) for cereal bars made from almonds of chichai, sapucaia and gurgueia. The mean value score for taste decreased from 6.92 to 5.61. Lower score for taste in cereal bars is due to the increasing level of Mung powder in as it gives bitter taste and legumes have after taste and beamy flavor. The results are in closer with the findings of Santos et al., (2011) for cereal bars made with jackfruit. The mean sensory score for texture decreased from 6.61 to 5.80. The highest mean score value for texture was found in control sample (6.61) while as lowest mean score for texture was found in 8% incorporation level of mung powder $T_4$ (5.80). This might be due to fiber in mung powder and may be due to high water absorption capacity of mung powder resulting in dehydration which may increase the cereal bar hardness because of increased 8% level of mung powder. Similar findings were presented by Agbaje et al., (2014) for cereal bars made from glutinous rice flakes and sunnah foods. In appearance of cereal bars, control sample got the highest score (6.66), while as the lowest mean score for appearance was found in $T_4$ sample (6.64). The decrease in appearance scores might be due to the pigment and lipid oxidation resulting in non-enzymatic browning.

The results were closer to the results of Shaheen et al., (2013) and others for date

based fiber enriched bars. The mean values for overall acceptability score of cereal bars indicated that $T_2$ has maximum 6.52 score. Overall acceptability was determined on the basis of quality scores obtained from the evaluation of color, texture, flavor and taste of the cookies. The data in Table-6 outlines that the majority of panelists accept the cookies made of $T_2$ level which has score of as 6.52 on 9 point scale. The overall acceptability for various treatments is in accordance with the findings of Al-Hooti et al., (1997) who reported overall acceptability score from 6.9 to 7.3. Agbaje et al. (2014) also reported similar results regarding the overall acceptability for cereal bars made from glutinous rice flakes.

**Table 4.** Fatty acids, Peroxide value, Browning index and Water activity of Rice based Cereal bars

| **Parameter** | **$T_0$ (0%)** | **$T_1$ (2%)** | **$T_2$ (4%)** | **$T_3$ (6%)** | **$T_4$ (8%)** |
|---|---|---|---|---|---|
| Water Activity (%) | 0.590±0.012 $^{a, C}$ | 0.58±0.012 $^{b, C}$ | 0.581 ± 0.012 $^{b, C}$ | 0.577±0.012 $^{c, C}$ | 0.573± 0.012 $^{c, C}$ |
| Fatty acids (%) | 1.05 ± 0.04 $^{e, B}$ | 1.06 ± 0.07 $^{d, B}$ | 1.07 ± 0.01 $^{c, B}$ | 1.09± 0.04 $^{b, B}$ | 1.11 ± 0.04 $^{a, B}$ |
| PeroxideValue ($meqO_2$/kg oil) | 5.92 ± 1 .04 $^{a, A}$ | 5.66 ± 1.03 $^{b, A}$ | 5.64 ± 1.03 $^{b, A}$ | 5.61 ± 1.01 $^{b, A}$ | 5.60 ± 1.01 $^{b, A}$ |
| Browning index (OD) | 0.07 ± 0.03 $^{e, D}$ | 0.08 ± 0.04 $^{d, D}$ | 0.10 ± 0.06 $^{c, D}$ | 0.12 ± 0.08 $^{b, D}$ | 0.14 ± 0.10 $^{a, D}$ |

Values are expressed as mean ± standard deviation. . Means having different letters within the same column differ significantly at $p < 0.05$.

**Table 5.** Color values of cereal bars

| **Treatments** | **L*(lightness)** | **a*(Redness)** | **b*(yellowish)** |
|---|---|---|---|
| $T_0$ | 49.92 ± 3.45 $^{b, A}$ | 10.96 ± 1.86 $^{c, A}$ | 50.69 ± 2.40 $^{a, A}$ |
| $T_1$ | 48.82 ± 3.02 $^{b, B}$ | 9.86 ± 1.66 $^{c, B}$ | 49.59 ± 2.14 $^{a, B}$ |
| $T_2$ | 47.72 ± 2.83 $^{b, C}$ | 8.76 ± 1.46 $^{c, C}$ | 48.49 ± 1.88 a, C |
| $T_3$ | 46.51 ± 2.57 $^{b, D}$ | 7.66 ± 1.26 $^{c, D}$ | 47.39 ± 1.62 $^{a, D}$ |
| $T_4$ | 45.52 ± 2.13 $^{b, E}$ | 6.56 ± 1.06 $^{c, E}$ | 46.29 ± 1.36 $^{a, E}$ |

Values are expressed as mean ± standard deviation. . Means having different letters within the same column differ significantly at $p < 0.05$.

**Table 6.** Effect of Mung powder on sensory perception of cereal bars

| **Treatments** | **Appearance** | **Taste** | **Colour** | **Texture** | **Overall Acceptability** |
|---|---|---|---|---|---|
| $T_0$ | 6.66±1.03 $^{b, A}$ | 6.92 ± 1.04 $^{a, A}$ | 6.64 ± 1.03 $^{b, A}$ | 6.61 ± 1.01 $^{b, A}$ | 6.39 |
| $T_1$ | 6.59 ± 1.02 $^{b, B}$ | 6.85 ± 1.03 $^{a, B}$ | 6.58 ± 1.02 $^{b, B}$ | 6.33 ± 1.04 $^{b, B}$ | 6.43 |
| $T_2$ | 6.33 ± 0.97 $^{c, C}$ | 6.70 ± 1.02 $^{a, C}$ | 6.40 ± 1.01 $^{b, C}$ | 6.24 ± 1.03 $^{d, C}$ | 6.52 |
| $T_3$ | 5.98 ± 1.03 $^{c, D}$ | 6.08 ± 1.02 $^{b, D}$ | 6.08 ± 1.02 $^{b, D}$ | 6.09 ± 1.01 $^{a, D}$ | 6.19 |
| $T_4$ | 5.54 ± 1.04 $^{c, E}$ | 5.61 ± 1.03 $^{b, E}$ | 5.62 ± 1.01 $^{b, E}$ | 5.80 ± 1.04 $^{a, E}$ | 5.97 |

Values are expressed as mean ± standard deviation. . Means having different letters within the same column differ significantly at $p < 0.05$.

### 3.6. Scanning electron microscopy (SEM) of rice based cereal bars

The micrographs of cereal bars are shown in Fig 1, 2 & 3. Samples stored under PP showed a spongy (Comb Like) appearance while the samples stored under PFP showed less sponginess. The formation of a gel like network in treated sampled stored under PFP is indicative of the agglomeration of the protein network. A more compact and dense structure is formed upon PFP which is evident from micrographs. The micrograph (Fig. 1A) of the control sample also had spongy appearance while the treatment sample packaged under PFP conditions had more compact appearance than all other formulations.

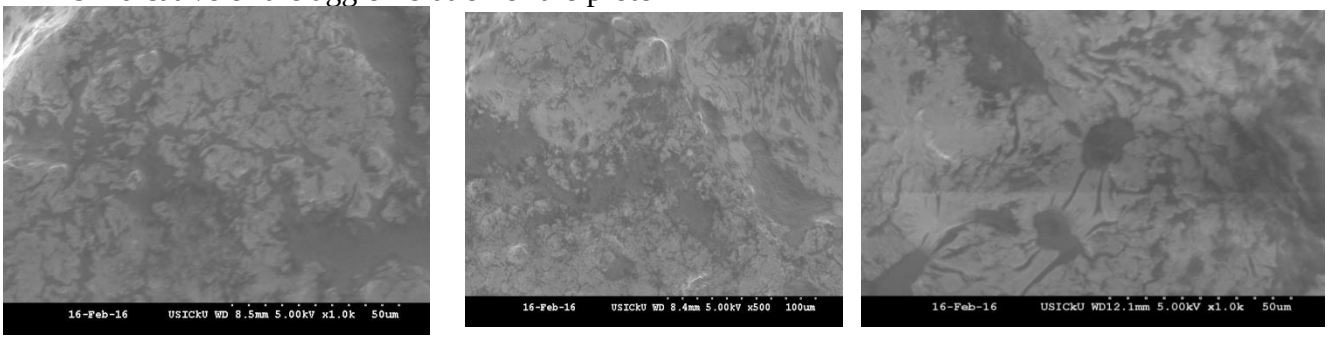


**Figure 1. (A) (B) (C)**

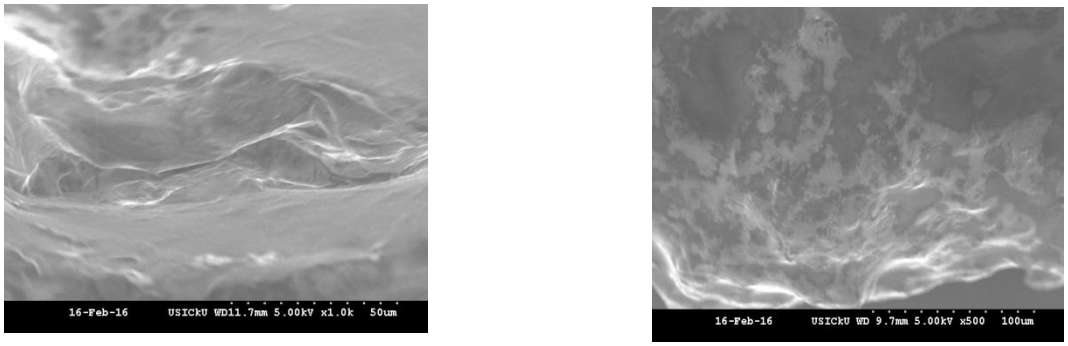


**Figure 2. (A) (B)**

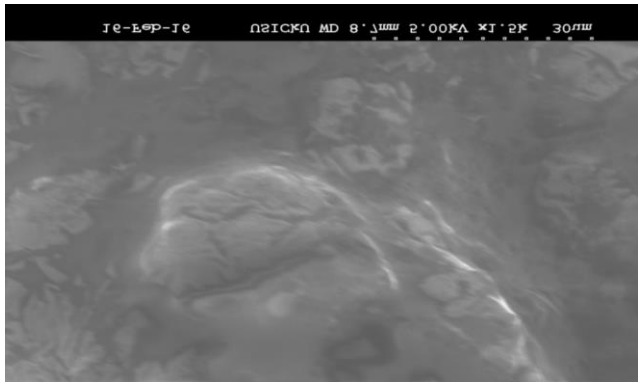

**Figure 3.**

Cereal-based foods are derived from grains that have a well-organized microstructure (Autio & Salmenkallio-Marttila, 2001). The microstructure determines the appearance and texture of protein fractions and the stability of the final product. Therefore it was important to study the effect of mung beans on the microstructure of cereal bars.

Micrographs of cereal bars had shown an extensive protein starch network holding starch granules intact. Also, it has been observed that there is excessive starch gelatinization in some areas which may be due to coarse flour of rice and mung bean powder. There has been seen less granular structure with starchy endosperm completely fused and had formed cavities with irregular boundaries. Such structural modifications had been visualized by Braadbaart et al., (2005) in wheat. It was also seen that the protein matrix in starch granules with very small air sacs which could be due to moisture retention by fiber in mung beans. However, in some areas prominent peaks had been observed embedded with air resulting in bulging of certain areas.

## 4. Conclusions

Incorporation of high levels of mung bean and rice in cereal bars is feasible. The supplemented products were accepted to consumers, however incorporation of high levels of supplements results in change in physico-chemical, sensory, and other characteristics.

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# COCONUT OIL BASED COOKIES FORTIFIED WITH BIO-CALCIUM: CHARACTERISTICS AND NUTRITIONAL COMPOSITIONS

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**ABSTRACT**

The main objective of this study was to develop coconut oil based cookie fortified with tuna bone bio-calcium (Bio-Ca) as healthy food rich in medium chain fatty acid and calcium. The impacts of Bio-Ca supplementation and replacement of shortening with coconut oil on the quality and sensory properties of cookie were determined. Colour, diameter, thickness as well as hardness of cookies varied with levels of Bio-Ca powder and coconut oil. Bio-Ca powder at 12% could be fortified into cookie using coconut oil as shortening replacer without the adverse effect on sensory properties. Scanning electron microscopy showed that the developed cookies had less porosity with denser structure, compared to that of the control. The developed cookie showed higher saturated fat, ash and protein contents but lower energy value, compared to the control. It was rich in calcium and phosphorous. Thus, it could serve as the mineral supplement, especially for calcium.

## 1. Introduction

Calcium (Ca) is a structural component of human body combined with phosphorus to comprise the mineral portion of bone and teeth (Weaver and Heaney, 1999). Ca also plays an important role in the treatment and prevention of Ca deficiency in patients. There is a numerous forms of Ca used for supplementation, particularly the purified Ca carbonate with a high Ca content (about 40%) (Hassan, 2015). However, only small portion of Ca can be absorbed, mainly associated with its precipitation in gastro-intestinal track. Food Ca must be dissolved in the acidic environment of the stomach and remained soluble (Gueguen and Pointillart, 2000). From total amount of absorbed Ca, 90% takes place in the intestines, in which the pH is slightly alkaline and reprecipitation hinders calcium uptake (Wasserman, 2004). Ca bound with proteins, so called bio-calcium (Bio-Ca), showed a high solubility under the neutral pH with a high Ca bioavailability, compared with inorganic counterpart (Stupar et al., 2009; Jung et al. 2006). Jung et al. (2006) found that the Bio-Ca from fish bone had the increased Ca solubility and bioavailability in ovariectomised rats. Recently, Bio-Ca powder from pre-cooked skipjack tuna bone has been produced and characterized (Benjakul et al., 2017). With the fine particle (17.07-20.29 μm) and negligible amount of volatiles of tuna bone Bio-Ca, it could be beneficially applied in foods, particularly snack products, for calcium fortification.

Cookies are flour-based products, which attract consumers owing to their various tastes, long shelf-life, relatively low cost and

availability for all consumer groups. Due to the increasing demand for health promoted natural products, there are several kinds of cookies fortified with the selected ingredient or supplement such as sunflower oil (Jacob and Leelavathi, 2007), soybean/pea protein (Bashir et al. 2015) and fructooligosaccharide (Handa et al., 2012). Coconut oil is one of the few foods that can be classified as health food from natural source. Coconut oil has been proven to provide a multitude of health benefits such as reducing inflammation and supporting almost all processes of the body (DebMandal and Mandal, 2011). It has a distinctive flavour and is mainly composed of three medium chain fatty acids (MCFAs), including caprylic acid, lauric acid and capric acid. Those MCFAs possess specific functional characteristics unlike long-chain fatty acids (LCFAs) found in other plant based oils (Yong et al., 2009). The fortification of Bio-Ca powder as well as the replacement of shortening with coconut oil could be of choice in production of cookie rich in MCFAs and Ca in bioavailable form. However, the level of both coconut oil and Bio-Ca could affect the quality and acceptability of consumers. This study aimed to develop coconut oil based cookie fortified with Bio-Ca at various levels, in which shortening was replaced by coconut oil at 65 and 100%. Physical, textural, sensory and nutritional characteristics of resulting cookies were evaluated in comparison to the typically made cookie.

## 2. Materials and methods

### 2.1. Materials

Palm oil shortening (50% palm oil, 26% hydrogenated palm kernel olein, 16% hydrogenated palm oil, 7% palm stearin and 0% trans-fat) (OP Kream Brand, Katevanich Industry Co., Ltd, Thailand), coconut oil (Naturel, Lam Soon (Thailand) Public Co., Ltd, Thailand), commercial wheat flour (Kite, United Flour Mill Public Co., Ltd, Thailand), icing sugar, backing powder, salt and peanut butter were purchased from a local market in Hat Yai, Songkhla, Thailand.

### 2.2. Preparation of Bio-Ca

Bio-Ca powder was prepared from pre-cooked skipjack tuna bone according to the method described by Benjakul et al. (2017). Briefly, bones of pre-cooked skipjack tuna (Songkla Canning Public Co., Ltd., Songkhla, Thailand) were longitudinally cut and cleaned to remove the remaining meat or other residues. Prepared bones were soaked in 2 M NaOH with a bone/solution ratio of 1:10 (w/v) at 50°C for 30 min to remove non-collagenous proteins. Bones were then dried using a laboratory scale rotary tray dryer at 50°C for 2 h and ground using a crushing mill (YCM-1.1E, Yor Yong Hah Heng, Bangkok, Thailand) to obtain particle sizes of approximately 3-4 mm. Ground bone was further soaked in hexane using a sample/solvent ratio of 1:10 (w/v) at 25°C and continuously stirred for 60 min. After draining, samples were allowed to stand at room temperature until dried and free of hexane odour. The bone sample was then bleached by soaking in 10 volumes of 2.5% (v/v) sodium hypochlorite for 30 min, followed by soaking in 10 volumes of 2.5% (v/v) hydrogen peroxide for 60 min. The sample was washed with running water for 5 min. The sample was dried in a rotary tray dryer at 50 ºC for 5 h and ground into the fine particles using a Ball Mill (PM 100, 127 Retsch GmbH, Haan, Germany). The obtained powder was sieved using a sieving machine (Vibratory Sieve Shaker analysette 3 Pro, FRITSCH GmbH, Deutschland, Germany), in which the particles having the sizes less than 75 µm were collected. The powder was referred to as "Bio-Ca powder".

### 2.3. Preparation of cookie

The cookie was prepared using the traditional technology and formulation. The batter formulation of control cookie was as follows (based on batter weight): 55.20% wheat flour, 20.7% icing sugar, 20.7% shortening, 2.0% peanut butter, 1.0% salt and 0.4% baking powder. For coconut oil based cookie, the

coconut oil was used to substitute the shortening at 65% and 100%. Bio-Ca powder was added into the batter at 8.5%, 12.0% and 15.5% (w/w). Firstly, all dry ingredients were mixed together in a dough mixer (KitchenAid casserole multifunctional 5k, KitchenAid, Benton Harbor, MC, USA) for 3 min. Thereafter all liquid ingredients including shortening and peanut butter, previously melted at 50 °C for 10 min, were added to the dry mixture and mixed at low speed for 3 min. The resulting cookie batter was sheeted to a thickness of a 2.0 cm and cut with a circular cookie cutter (diameter: 2.4 cm). The shaped cookie batter was baked in an electric oven (Mamaru MR-1214, Mamaru (Thailand) Co., Ltd., Bangkok, Thailand) at 180 °C for 10 min. After baking, cookies were allowed to cool at room temperature for 1 h before analyses. The cookies prepared using the mixed lipid were referred to as "ML cookies", while those made using 100% coconut oil were termed as "CO cookies".

## 2.4. Analyses

### *2.4.1. Colour*

The colour of samples was determined using a colourimeter (ColorFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system, including L*, a* and b*, representing lightness, redness/greenness and yellowness/blueness, respectively. Total difference of colour (ΔE*) and chroma difference (ΔC*) were calculated as described by Takeungwongtrakul et al. (2015)

$$\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (1)$$

$$\Delta C^* = \sqrt{\Delta a^{*2} + \Delta b^{*2}} \quad (2)$$

where ΔL*, Δa*and Δb*are the differences between the corresponding colour parameter of the sample and that of control cookie.

### *2.4.2. Measurement of hardness*

Hardness of cookies was determined by a texture analyser (Stable Micro Systems, Godalming, Surrey, UK) using a test speed of 10 mm/s required to cut the cookies with a load cell of 50 kg. A special pasta blade and plate (probe TA 47, 60 mm x 20 mm) were used to imitate the biting action of a tooth. The maximum force required to break cookies was calculated for each sample. Ten measurements were made for each sample.

### *2.4.3. Physical measurement of cookies*

Cookies were evaluated for thickness (cm), diameter (cm) and spread ratio. Five cookies were used for the evaluation of each sample. The spread ratios were determined according to Chang et al. (2014). Spread ratio was calculated by dividing the diameter by thickness of cookies.

### *2.4.4. Sensory evaluation*

Sensory evaluation was performed by 50 untrained panellists, who were familiar with the consumption of cookies. Panellists were asked to evaluate for appearance, colour, odour, texture, taste and overall likeness using a nine-point hedonic scale, in which a score of 1 = not like very much, 5 = neither like nor dislike and 9 = like extremely. The samples were labelled with random three-digit codes. Panellists were instructed to rinse their mouth with water after each sample evaluation. The order of presentation of the samples was randomised according to ''balance order and carry-over effects design'' (Meilgaard et al., 2006).

## 2.5. Determination of chemical composition and energy value

The sample was analysed for moisture, protein, fat and ash contents using analytical method No. of 925.45(A), 981.10, 948.15 and 923.03, respectively (AOAC, 2002). Water activity (aw) was measured using a water activity meter (4TEV, Aqualab, Pullman, WA, USA). The energy, total carbohydrate, total sugar, saturated fatty acid, polyunsaturated fatty acid, monounsaturated fatty acid and trans-fat contents were estimated using compendium of methods for food analysis (Department of Medical Sciences and National Bureau of Agricultural Commodity and Food Standards, 2003).

### 2.6. Determination of mineral

Inductively coupled plasma optical emission spectrometer (ICP-OES) (Optima 8000, Perkin Elmer Instrument, Waltham, MA, USA) was used for determination of Ca and P in samples as per the method of Feist and Mikula (2014). The wavelengths used for Ca and P detection were 317.933 and 213.617 nm, respectively.

### 2.7. Scanning electron microscopy

Scanning electron microscopy (SEM) was used to examine the microstructure of cookies. The samples were mounted on individual bronze stubs and sputter-coated with gold layer. The specimens were visualised with a scanning electron microscope (LEO 440i, Oxford, UK) at an acceleration voltage of 15 kV and 5-10 Pa pressure.

### 2.8. Statistic analysis

Experiments were run in triplicate using three lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Physical characteristics and hardness of cookies

#### *3.1.1. Colour*

Surface colour of cookie containing coconut oil at 65% substitution of shortening (ML cookie) or 100% coconut oil (CO cookie) incorporated with tuna bone Bio-Ca powder at various levels in comparison with the control cookie is shown in Table 1. For ML cookies, surface had the lower L* value as the level of tuna bone Bio-Ca powder was above 8.5% ($P<0.05$), compared with that of control (shortening based cookie without Ca fortification). Furthermore, all CO cookies showed the darker surface ($P<0.05$) than the control as indicated by lower L* values. It was noted that CO cookie with 15.5% Bio-Ca powder showed the lowest L* value ($P<0.05$). For a* value, the lowest value was obtained for the control ($P<0.05$). For both ML and CO cookies, a* values increased with increasing level of Bio-Ca powder added ($P<0.05$). The similar a* value was found between ML and CO cookies when Bio-Ca powder at the same level was incorporated ($P>0.05$). The increase in b* value of ML cookie was observed as the Bio-Ca level increased ($P<0.05$). There was no difference in b* value of CO cookies in all Bio-Ca levels used ($P<0.05$). Overall, the cookie surface turned to be browner in colour, when Bio-Ca powder and coconut oil were used at higher proportion. The increases in $\Delta E^*$ and $\Delta C^*$ values of both cookies were also found as the amount of Bio-Ca powder increased, especially for ML cookies ($P<0.05$). Generally, CO cookies had the higher $\Delta E^*$ and $\Delta C^*$ values, compared with ML cookies when the same Bio-Ca powder was added ($P<0.05$). Tuna bone Bio-Ca powder used in the present study was creamy whitish in colour (Benjakul et al., 2017). The shortening and coconut oil used plausibly exhibited the different physical property, governed by solid fat content (SFC), which represented the amount (%) of solid fat present in the oil at any given temperature. SFC was influenced by types of triacylglycerols in the oil (Liang et al., 2014). At room temperature (25-28 °C), shortening was in solid state with high plasticity, while coconut oil was in liquid form with low proportion of crystal phase. SFC can influence appearance, flavour release, melt rate, shelf-life and stability of fat based food products (Liang et al., 2014). Solid state of shortening might be associated with the light scattering of products as indicated by the higher lightness. Although coconut oil was liquid in nature at room temperatures, the consistent batter could be formed after the addition of Bio-Ca powder. This helped in molding the cookies prior to baking. For Bio-Ca powder, the proteins in powder might undergo Maillard reaction during baking at high temperature. This could lead to the increases in a*- and b*-values.

### *3.1.2. Diameter, thickness and spread ratio*

Diameter, thickness and spread ratio of ML and CO cookies containing Bio-Ca powder at different levels are presented in Table 2. All cookies showed the lower diameter (2.44-2.71 cm) than the control (2.78 cm), except that of ML cookies fortified with 8.5% Bio-Ca powder ($P>0.05$) which had similar diameter to that of control. The diameter of cookie decreased as the level of Bio-Ca powder used increased ($P<0.05$). On the other hand, the increases in thickness of cookies were noticeable as the levels of Bio-Ca added increased ($P<0.05$). ML cookies showed the lower diameter with the higher thickness, compared to CO cookies at the same level of Bio-Ca powder used ($P<0.05$). The result suggested that the different proportions of coconut oil used, the presence of shortening as well as the level of Bio-Ca powder had the influence on diameter and thickness of resulting cookies. Those factors caused the varying of spread ratio of the resulting cookies. Spread ratio value decreased with increasing level of Bio-Ca powder ($P<0.05$) for both ML and CO cookies. CO cookies generally showed the higher spread ratio than ML cookies, when the same level of Bio-Ca was added ($P<0.05$). Hassan (2015) used the chicken eggshell powder for Ca fortification in biscuits and found that the thickness increased with the decreased spread ratio as the level of eggshell powder increased. Jacob and Leelavathi (2007) reported that the cookies containing sunflower oil had relatively higher spread value, compared with those used margarine and hydrogenated fat. Coconut oil with the liquid state showed the flow behaviour. Nevertheless, Bio-Ca powder could increase consistency of the resulting mixture, in which the cookies could be moulded before baking. During heating, the oil could flow to some extent as indicated by higher spread ratio. The result indicated that fat/oil type and level of Bio-Ca powder directly determined the physical characteristics, including diameter, thickness and spread ratio of cookies.

### *3.1.3. Hardness*

Hardness of ML and CO cookies added with tuna bone Bio-Ca powder at various levels are shown in Table 2. Increases in hardness were found as the Bio-Ca level increased for both cookies ($P<0.05$). Hardness of the control was 30.54 N. The cookies added with Bio-Ca powder at the highest level (15.5%) showed the highest hardness ($P<0.05$). At the same level of Bio-Ca used, ML cookies showed the lower hardness than CO cookies ($P<0.05$). Due to the fine particle of tuna bone Bio-Ca powder (17.07-20.29 μm) (Benjakul et al., 2017), it could fill the void of cookie's structure, thus providing the denser and harder texture. Hassan (2015) found that the addition of Ca from different sources led to smaller size pores or air cells in the puffed crisp biscuit. This was related with the harder texture. The oil used for preparing cookie also influenced the texture of final product (Mamat and Hill, 2014). Jacob and Leelavathi (2007) studied the effect of fat types on cookie dough and cookie quality. Cookies containing liquid oil had a relatively harder texture, compared to bakery and hydrogenated fat. The ratio of the solid phase to the liquid phase or SFC is an important factor determining the functionality of the fat/shortening in the dough. Cookies made from sunflower oil had the hardest texture, whilst those containing bakery fat, margarine and hydrogenated fat were softer. Soft fat lowered the breaking strength of the cookies (Jacob and Leelavathi, 2007). In the present study, the lower hardness of ML cookies containing 35% shortening was attained, compared to CO cookies. In addition, the liquid oil could be dispersed upon mixing throughout the cookie batter in the form of globules that are less effective in their shortening and aerating action (Hartnett and Thalheimer, 1979). ML cookies might have the lower incorporated air. As a result, more compact structure was formed, leading to the increased hardness of CO cookies. Bio-Ca powder at higher levels caused the increased hardness of cookies. Those particles might construct the stronger matrix as well as strengthen the cookies via filler effect.

### 3.2. Sensory properties

The effect of addition of tuna bone Bio-Ca powder at various levels on sensory properties of ML and CO cookies is presented in Table 3. There was no difference in appearance, flavour, texture and mouth feel likeness score between control, ML and CO cookies ($P<0.05$), except for CO cookies added with 15.5% Bio-Ca powder, which showed the lowest score ($P<0.05$). Moreover, the incorporation of Bio-Ca powder at the highest level (15.5%) reduced the likeness score for colour, taste and overall attributes of both cookies. This was related with the darker colour (Table 1) and increased hardness (Table 2) of cookie, especially for CO cookies. It was noted that the maximal level of Bio-Ca powder, which showed no detrimental effects on sensory properties of resulting cookies was found at 12% for CO cookies. Likeness scores for all attributes tested of CO cookies containing 12% Bio-Ca were comparable to those of the control ($P>0.05$). It was reported that the Bio-Ca powder could be used as Ca dietary supplement in bread, pizza, spaghetti or biscuit with small changes in physical and sensory characteristics (Brun et al., 2013; Hassan, 2015). The use of coconut oil as shortening replacer could be of health benefit. CO cookie added with 12% Bio-Ca powder was then selected to study its chemical characteristics and nutritional value in comparison with the control.

**Table 1.** Color of cookies prepared using mixed lipids or coconut oil with addition of Bio-Ca powder at various levels

| Characteristics | Samples | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | ML-8.5* | ML-12 | ML-15.5 | CO-8.5 | CO-12 | CO-15.5 |
| Color | | | | | | | |
| $L^*$ | 76.28±0.69a | 76.47±0.28a | 73.34±0.33b | 72.31±0.50c | 71.87±0.42c | 72.04±0.26c | 69.19±0.77d |
| $a^*$ | 4.52±0.07d | 5.18±0.16c | 5.93±0.27b | 6.76±0.23a | 5.35±0.14c | 6.12±0.29b | 7.45±0.33a |
| $b^*$ | 23.92±0.39d | 25.94±0.13c | 27.79±0.53b | 28.80±0.35a | 28.94±0.49a | 28.67±0.31a | 30.93±0.36a |
| $\Delta E^*$ | - | 1.88±0.04d | 2.18±0.30c | 2.83±0.21b | 2.62±0.29b | 2.42±0.15b | 3.65±0.22a |
| $\Delta C^*$ | - | 1.64±0.13e | 3.49±0.45d | 4.81±0.51b | 4.84±0.31cd | 5.11±0.49bc | 8.23±0.76a |

Control: shortening (100%).
ML: mixed lipids (35% shortening+65% coconut oil).
CO: coconut oil (100%).
*Numbers denote the level of Bio-Ca powder (% of batter weight).
Different lowercase letters in the same row indicate significant difference ($P < 0.05$).

**Table 2**. Characteristics and hardness of cookies prepared using mixed lipids or coconut oil with addition of Bio-Ca powder at various levels

| Samples | Diameter (cm) | Thickness (cm) | Spread ratio | Hardness (N) |
|---|---|---|---|---|
| Control | 2.78±0.05a | 1.51±0.01d | 1.83±0.02c | 30.54±2.38b |
| ML-8.5* | 2.78±0.07a | 1.44±0.03e | 1.93±0.07b | 15.85±1.09d |
| ML-12 | 2.46±0.04d | 1.52±0.01d | 1.60±0.02e | 21.29±1.71c |
| ML-15.5 | 2.44±0.04d | 1.64±0.01a | 1.48±0.02f | 34.28±1.79a |
| CO-8.5 | 2.71±0.06b | 1.29±0.02f | 2.10±0.05a | 22.08±1.53c |
| CO-12 | 2.67±0.02b | 1.57±0.02c | 1.72±0.03d | 30.64±1.92b |
| CO-15.5 | 2.61±0.03c | 1.60±0.01b | 1.64±0.03e | 37.92±1.08a |

Control: shortening (100%).
ML: mixed lipids (35% shortening+65% coconut oil).
CO: coconut oil (100%).
*Numbers denote the level of Bio-Ca powder (% of batter weight).
Different lowercase letters in the same column indicate significant difference ($P < 0.05$).

**Table 3.** Sensory properties of cookies prepared using mixed lipids or coconut oil with addition of Bio-Ca powder at various levels

| Samples | Likeness score | | | | | | |
|---|---|---|---|---|---|---|---|
| | Appearance | Color | Flavor | Texture | Mouth feel | Taste | Overall |
| Control | 7.75±0.74a | 7.79±0.72a | 7.75±0.85a | 7.88±1.03a | 7.88±0.80a | 7.88±0.99a | 7.58±0.83ab |
| ML8.5* | 7.62±0.65ab | 7.50±0.59ab | 7.25±1.03ab | 7.42±0.97ab | 7.29±1.00ab | 7.33±1.17abc | 7.79±0.66a |
| ML-12 | 7.46±0.83ab | 7.58±0.78ab | 7.17±1.09ab | 7.58±1.02ab | 7.50±1.06ab | 7.62±1.01abc | 7.17±0.87bc |
| ML-15.5 | 7.63±0.71ab | 7.25±0.94b | 7.62±0.92ab | 7.38±0.97ab | 7.33±1.13ab | 7.08±0.97bc | 7.04±0.95c |
| CO-8.5 | 7.88±0.68a | 7.79±0.78a | 7.50±1.10ab | 7.67±0.76ab | 7.50±0.78ab | 7.50±0.93abc | 7.71±0.69a |
| CO-12 | 7.50±0.78ab | 7.42±0.83ab | 7.25±0.90ab | 7.50±0.93ab | 7.67±0.76ab | 7.71±0.81ab | 7.33±1.05abc |
| CO-15.5 | 7.21±0.72b | 7.12±0.85b | 7.08±1.10b | 7.12±0.99b | 7.08±1.06b | 7.04±1.20c | 7.00±0.88c |

Control: shortening (100%).
ML: mixed lipids (35% shortening+65% coconut oil).
CO: coconut oil (100%).
*Numbers denote the level of Bio-Ca powder (% of batter weight).
Different lowercase letters in the same column indicate significant difference ($P < 0.05$).

**Table 4.** Chemical composition and energy value of the control cookie and the developed cookie

| Compositions/energy value | Samples | |
|---|---|---|
| | Control | CO-12 |
| Moisture (g/100g) | 3.36±0.31a | 2.71±0.36b |
| Protein (g/100g)** | 7.60±0.09b | 9.95±0.67a |
| Total fat (g/100g) | 24.33±1.26a | 23.97±1.08a |
| Saturated fat (g/100g) | 12.44±0.97b | 16.81±0.54a |
| Monounsaturated fat (g/100g) | 8.05±0.22a | 3.50±0.74b |
| Polyunsaturated fat (g/100g) | 2.63±0.08a | 1.46±0.27b |
| Total carbohydrate (g/100g) | 64.02±1.53a | 52.55±1.69b |
| Total sugar (g/100g) | 21.77±1.41a | 13.62±1.03b |
| Ash (g/100g) | 1.34±0.53b | 10.17±0.94a |
| Calcium (g/100g) | 0.04±0.00b | 2.94±0.02a |
| Phosphorous (g/100g) | 0.11±0.00b | 1.18±0.01a |
| Sodium (g/100g) | 0.37±0.00b | 0.39±0.00a |
| Trans-fat (mg/100 g) | nd | nd |
| Energy value (kcal/100 g) | 505.45±25.48a | 465.73±15.67b |

** The conversion factor is 6.25.
Control: shortening (100%) without Bio-Ca powder.
CO-12: coconut oil (100%) with 12% Bio-Ca powder.
nd: not detected.
Different lowercase letters in the same row indicate significant difference ($P < 0.05$).

### 3.3. Chemical characteristics and nutritional value of cookies

The chemical compositions of the developed cookies (CO cookies fortified with 12.0% Bio-Ca powder) in comparison with the control (cookie prepared using shortening) are shown in Table 4. The major component of both samples was carbohydrate (52.22-64.02%). However, the lower carbohydrate (52.55%) and sugar (13.62%) contents were found in the developed cookie, compared with those of the control ($P<0.05$). It was noted that the developed cookie had the lower moisture content (2.71 %), compared with that of the control (3.36%). In

addition, the significant increases in protein and ash contents were observed in the developed cookie. Tuna bone Bio-Ca powder consisted of 24.26% protein and 72.20% ash (Benjakul et al., 2017). With addition of Bio-Ca powder, these components in the final product were increased. Benjakul et al. (2017) reported that pre-cooked tuna bone Bio-Ca powder was a good source of Ca and phosphorous (P), present as hydroxyapatite along with collagenous proteins. The marked increases in Ca and P contents were attained in the developed cookie. This result suggested that tuna bone Bio-Ca powder could be effectively used for Ca fortification in cookie.

The replacement of shortening by coconut oil in cookie showed the noticeable effect on chemical composition of resulting cookie, especially fat compositions. CO cookie fortified with Bio-Ca powder had the similar total fat content (23.37%) to the control (24.33%). Nevertheless, the higher content of saturated fat with lower mono- and poly-unsaturated fats was obtained in the former. Normally, coconut oil is abundant in medium chain saturated fatty acids (MCFAs) (DebMandal and Mandal, 2011). Patil et al. (2016) found that virgin coconut oil extracted from different maturity stages showed the similar fatty acid profile, in which lauric acid (C12:0) was a major fatty acid (49.74-51.18 g/100g). The coconut oil used in the present study contained saturated fat more than 90%. The shortening used was produced from palm oil, which was high in long chain fatty acid content, mainly palmitic (C-16:0) acid and oleic acid (C-18:1) (Lida et al., 2002). Palm oil consists of trisaturated (S3) (mainly PPP), disaturated (S2U) (mainly POP), and monosaturated (U2S) (mainly POO) triacylglycerides, where P is palmitic acid and O is oleic acid (Timms 1985). The shortening used also contained hydrogenated palm kernel olein (26%) and hydrogenated palm oil (16%). During shortening process, hydrogenation more likely reduced the degree of unsaturation to some degree (O'Brien, 2004). These results indicated that the fat composition of cookie was governed by the fat type used for cookie preparation.

The trans-fat content of both control and CO cookie fortified with Bio-Ca powder was also investigated. It was noted that trans-fat was not detected in both samples tested (Table 4). This result suggested that trans-fat was not formed, especially during baking at 180 °C for 10 min. The energy values of cookie (Table 4) were calculated using the Atwater factor of 4, 9 and 4 kcal/g for calculation from protein, fat and carbohydrate, respectively (Prokopov et al., 2015). The energy value of the developed cookie was lower than that of the control ($P<0.05$). Since carbohydrate was the major component of cookie, the lower carbohydrate content of the developed cookie yielded the lower energy, compared with that of the control. The results suggested that the use of tuna bone bio-calcium powder for Ca fortification as well as the replacement of shortening with coconut oil in cookie rendered the final product with the improved nutritional value and functional aspect.

### 3.4. Structure of cookies

Scanning electron microscopic images of the internal cross-sectional area of cookies are shown in Figure 1. The control cookie had an open structure with air cells distributed throughout the cookie matrix. This led to the rough crumb and porous structure. The distinctive difference in the internal structure of the developed cookie was observed, compared to that of the control. The less air cell with the higher uniformity was found in the developed cookie. This dense and smooth matrix was related with lower diameter, higher thickness as well as lower spread ratio (Table 2), compared with those of control. The Bio-Ca powder added could fill the void or air cell in the cookie crump and provided the denser structure. Moreover, the distribution of Bio-Ca power throughout the batter matrix might interrupt the aeration property of cookie batter. Coconut oil used was the liquid, which could not entrap and retain the considerable volume of air. Conversely, the shortening used in the control cookie had more solid fat content with high plasticity, which

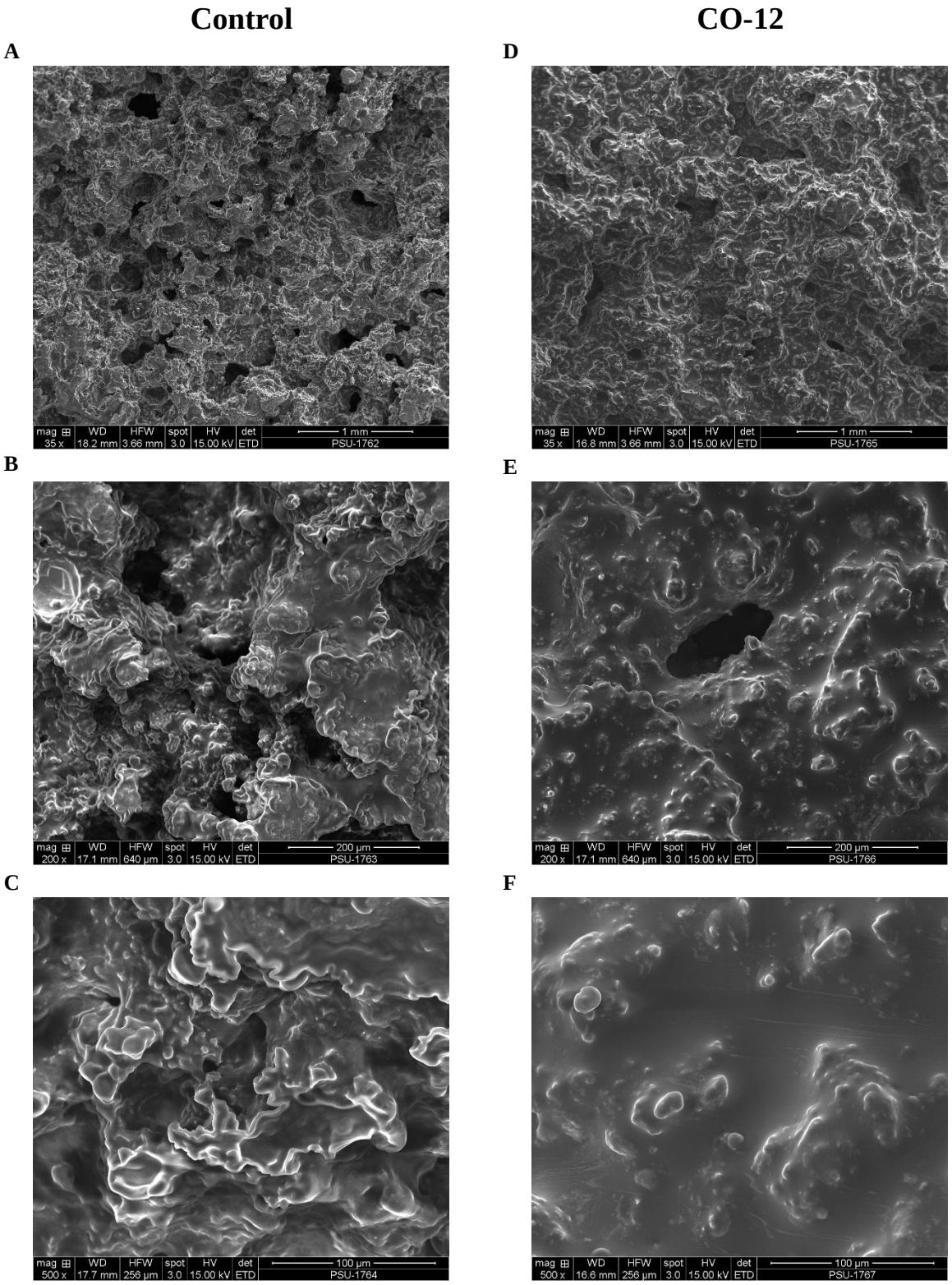


**Figure 1.** Scanning electron microscopic photographs of internal structure of the control cookie (A, B, C) and the developed cookie. Control: 100% shortening used for preparing cookie without bio-calcium fortification. CO-12 cookie: 100% replacement of shortening with coconut oil and fortification of 12% bio-calcium powder. A and D: 35x magnification, B and E: 200x magnification, C and F: 500x magnification.

could trap and hold air bubbles into the cookie batter throughout mixing and baking processes. The fat crystals in semi-solid fat could be enveloped in a protein membrane, which allowed the fat crystals attach to air bubbles. These fat crystals were melted during baking and the protein membrane was incorporated into the surface of air bubbles as they expanded, resulting in the rupture resistance of air cells (Manley, 2000). These results suggested that use of Bio-Ca powder for fortification and the replacement of shortening with coconut oil in cookie had the impact on internal structure of resulting cookie, which was associated with characteristics of cookie.

## 4. Conclusions

Incorporation of Bio-Ca powder and replacement of shortening with coconut oil had the impact on the cookie quality and sensory properties. Bio-Ca powder at 12% could be fortified into cookie prepared using coconut oil instead of shortening without the adverse effect on sensory properties. Therefore, cookies with health benefit could be prepared and served as an alternative for health conscious consumers.

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# EFFECT OF BALL MILLING ON THERMAL, MORPHOLOGICAL AND STRUCTURAL PROPERTIES OF STARCHES FROM *Zingiber officinale* AND *Dioscorea sp*.

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**ABSTRACT**

Ginger and yam can be considered alternative sources of starch with specific properties, which can be modified through physical modifications, considered efficient and environmentally friendly. Thus, this study aimed to modify starches isolated from *Zingiber officinalle* and *Dioscorea* sp through ball milling at different times, verifying its effects on the thermal, morphological and structural properties. After milling treatment an increase in the thermal stability was observed for ginger and yam starches with the increase of milling time. A decrease in the gelatinisation parameters for two sources of the ball-milled starches was identified, which was related to the weakening of amylopectin chains and amylose depolymerisation. A decrease in the size of both starch granules was obtained, with the appearance of surface fissures. The degree of relative crystallinity decrease with the higher exposition to ball milling, reflecting in an amorphous state, although the diffraction pattern has not changed for both starches. Slight changes can be observed by Fourier transform infrared spectra due the damages caused by milling process.

## 1.Introduction

Starch is one of the most important and abundant carbohydrates available in nature, in addition, it presents low cost even after the extraction process. This natural biopolymer is composed of two polysaccharides: amylose considered a mostly linear macromolecule, and amylopectin, a highly ramified one. Starch represents an important input for industries in different sectors such as food, textile, pharmaceutical, among others. Due to the wide application, industries are looking not only for new process technologies, but also for new sources of starch with different properties that make its production feasible (Lawal et al., 2005; Vieira and Sarmento, 2008).

An alternative source of starch is from ginger, with a starch content around 13-18% according to Wang et al. (2008). Ginger is a plant belonging to the family of *Zingiberaceae*, originated in Asia and considered a popular spice used mainly in India for medicinal purposes. It can also be found in tropical and subtropical areas such as China, Indonesia and Nigeria (Madeneni et al. 2011; Sukhija, Singh and Ria, 2016; Li et al., 2016). Another source of starch of interest is from yam (*Dioscorea* sp.), which is a monocotyledonous herbaceous climbing plant. Around eight genera are known worldwide, with approximately 600 species, 95% belonging to the genus *Dioscorea* (Hornung et al, 2016). These tubers may have

different names depending on where they are grown. They are cultivated mainly in tropical and subtropical regions, and because of their easy handling they have become very consumed by millions of people in Africa, Asia and South America (Ramos et al., 2014; Zhu, 2015).

In addition to the search for new sources of starch, polysaccharides modifications are interesting, because in their native form, they can present some technological limitations. The main treatments applied on starch granules are: enzymatic, physical or chemical modifications, with the aim of obtaining starches with desirable and specific properties in order to meet a range of industrial applications (Park et al., 2009; Shima et al., 2015). Thus, several studies are carried out to investigate special starch characteristics obtained after these treatments. Although chemical modification has been widely applied in industry, physical modification regards to a market trend when used in natural products. It has potential to alter the functionality of starch at low cost in addition to being environmentally friendly (Zavareze and Dias, 2011).

Ball milling is a process that uses thermal and mechanical energy to modify the physicochemical properties of a material, presenting a high efficiency. Therefore, it has been largely used to grind minerals, foods, drugs, chemical products and construction material (Shi et al., 2015). In this type of treatment, the granular structure of the starch can be destroyed, with the formation of an amorphous material, with particles of smaller diameters (Sanguanpong et al., 2003). Consequently, the conformation of double helices is altered, promoting a decrease in relative crystallinity. Moreover, the fragmentation of the amylopectin chains improves the dilatation capacity, aiding the gelatinisation process (Alcázar-Alay and Meireles, 2015). Changes in the solubility, thermal and morphological properties, as well as in the digestion of starches can also occur (Moraes, Alves and Franco, 2013; He et al., 2014).

Therefore, this study aimed to modify starches isolated from ginger (*Zingiber officinalle*) and white yam (*Dioscorea* sp) through ball milling at different times (20, 30 and 40 min), verifying the results of this modification by thermal, morphological and structural analysis.

## 2. Materials and methods

### 2.1. Materials

Ball milling was used at Paulista State University Júlio de Mesquita Filho (Bauru, SP, BR). Extraction and instrumental analysis were made at Laboratories of Food Engineering Department and at Complex of Multi-Users Laboratory of State University of Ponta Grossa (UEPG).

### 2.2. Starch extraction

Starches were obtained from roots of ginger and yam according to the methodology describe by Costa et al. (2013).

### 2.3. Ball Milling modification

Starches from ginger (*Zingiber officinale*) and white yam (*Dioscorea* sp), were submitted to abrasive milling in a vibrating mill MM 400 (Retsch, Germany). The milling process was performed with the following parameters: sample mass about 0,6 g; milling time of 20, 30 and 40 minutes; and a milling frequency of 30 Hz. The stainless steel sample holder has a capacity of 10 mL, containing two balls of same material and diameter (5 mm), which remain in contact with the sample throughout the treatment.

The native and modified samples of ginger and white yam starches were coded as follows: (a) native ginger starch, and modified with ball milling during: 20 min (a1); 30 min (a2) and 40 min (a3); (b) native yam starch and modified with ball milling

during: 20 min (b1); 30 min (b2) and 40 min (b3).

## 2.4. Thermal analysis

### *2.4.1 Differential scanning calorimetry (DSC)*

Each sample submitted to the DSC analysis was mixed with water (in the proportion of starch:water, 1:4) and kept at rest for 1 h for the granules swelling. The analysis was performed following the procedure described by Colman, Demiate and Schnitzler (2014).

The instrument used was the DSC Q200 (TA Instruments, USA). The equipment was previously calibrated with Indium standard, 99% purity, (m.p. = 156.6 ºC; ΔH = 28,56 J $g^{-1}$) The aim of this analysis was to obtain the gelatinisation parameters of the samples, then the following conditions were employed: sample mass of 2.5 ± 0,5 mg, heating rate of 10 °C $min^{-1}$, synthetic air atmosphere with a flow rate of 50 mL $min^{-1}$, with a temperature range from 30 to 100 °C. The results were analysed with the aid of the software Universal Analysis 2000.

### *2.4.2. Simultaneous Thermogravimetry and Differential thermal analysis (TG-DTA)*

The TG-DTA curves were obtained according to the methodology described by Malucelli et al. (2015), using α-alumina crucibles for each sample (mass around 7 mg) in the equipment DTG-60 (Shimadzu, Japan), previously calibrated with calcium oxalate, using synthetic air atmosphere with flow rate of 100 mL $min^{-1}$. The heating rate was 10 °C $min^{-1}$ in the range of 30 to 600 °C. The software used to obtain the results was TA-60 WS.

## 2.5. Morphological analysis

### *2.5.1. Field emission gun - Scanning electron microscopy (FEG-SEM)*

The diameter and shape of the starch granules were verified using a field emission electronic microscope MIRA 3 (Tescan, Czech Republic), using the following parameters: the electrons beam tension was 15 kV in the field emission gun, generated by a lamp with tungsten filament. The samples were pulverised over a carbon tape, following by the metallisation process (120 s, 40 mA) with gold and palladium, to promote the passage of electrons, according to Bet et al. (2016).

### *2.5.2. Fourier Transform Infrared Spectroscopy (FTIR)*

According to Ji et al. (2015), the conditions used for this analysis were: an average of 64 scans at 4 $cm^{-1}$ of resolution, in the transmittance mode. The samples were prepared in a press, as pellets, consisting of 2 mg of sample in 100 mg of KBr. The wave number interval was 400 to 4000 $cm^{-1}$. It was used for this analysis the FT-IR 8400 (Shimadzu, Japan).

## 2.6. Structural analysis

### *2.6.1. X-ray powder diffractometry (XRD)*

The samples were put in a glass support and analysed in the X-ray diffractometer Ultima 4 (Rigaku, Japan), exposed to CuKα radiation (λ = 1.542 Å), submitted to 40 kV and 30 mA, with scattered radiation detected from the angles of $5º \leq 2\theta \leq 50º$, with a scanning speed of 2° $min^{-1}$ and a step of 0.02°. The diffractograms were treated in the software Origin 6.1 (OriginLab, USA), and the degree of relative crystallinity of the starches was calculated using the Equation 1 bellow (Colman, Demiate and Schnitzler, 2014):

$$Xc = \frac{Ap}{Ap+Ab} \times 100 \qquad (1)$$

Where: $X_c$ = relative crystallinity; $A_p$ = peak area; $A_b$ = basis area which refers to amorphous area of diffractograms.

### 2.7. Statistical analysis

All the analysis were made in triplicate, except otherwise stated, such as the TG-DTA, which was made in duplicate. The results were denoted in average ± standard deviation. Due the small numbers of repetition (n=3), the normality of the samples were assumed. The Brown-Forsythe's test was realized to verify the homoscedasticity of the variances (α = 95%). When observed homoscedastic variances, the results were compared using One-Way ANOVA, with α = 95%, in order to verify significant differences. Then, Tukey's HSD post-hoc test (α = 95%) was realized in order to elucidate where the differences occurred. The inferential statistics was realized using the software Statistica 13.2 (Dell, USA).

## 3.Results and discussions

### 3.1. Thermoanalytical techniques

#### *3.1.1.Differential scanning calorimetry*

It was noted (Figure 1) that the endothermic event readily identified for native ginger and white starch samples (samples ‘a’ and ‘b’) became always lower for starches after ball milling.

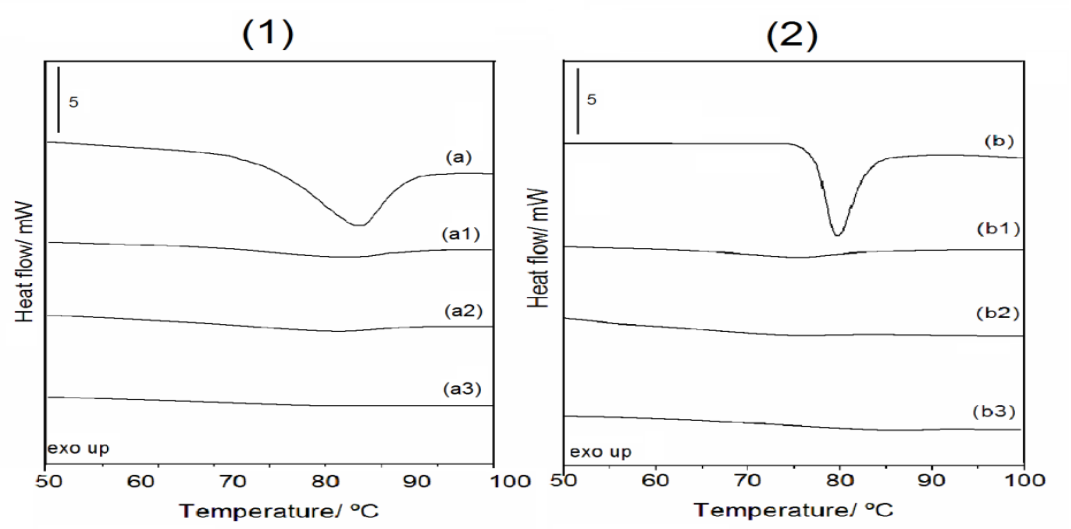


**Figure 1**. Differential scanning calorimetry curves of the native and modified starches from ginger (1) and white yam (2).

Ball milling compromises the crystalline structure of the starch, in an effect known as mechanical activation, associated with the friction, collision or shear, for example. During this modification, the temperature may also increase due to the frictional forces between the starch granules or between the small steel balls and the granules (Shi et al., 2015).

Thus, ball milling may cause damage to the granules, especially in the amylopectin chains, as well as observed by XRD, making gelatinisation difficult or shifting the thermal event, as identified for yam and ginger starch in the three times of treatment.

Moraes, Alves and Franco (2013) also reported broader endothermic peaks after the ball milling process, which were attributed to greater heterogeneity of the crystals. Therefore, the gelatinisation temperature range may vary according to the heterogeneity of the crystals. The transitions temperatures and gelatinisation enthalpy for all the samples can be identified in Table 1:

**Table 1.** Results of the differential scanning calorimetry (DSC)

| Sample | | DSC gelatinisation results | | | |
|---|---|---|---|---|---|
| | $T_o$ (ºC) | $T_p$ (ºC) | $T_c$ (ºC) | $T_c - T_o$ (ºC) | $\Delta H_{gel}$ (J $g^{-1}$) |
| **(a)** | 80.01 ± 0.05$^a$ | 87.73 ± 0.21$^c$ | 92.01 ± 0.16$^a$ | 12.00 | 15.15 ± 0.39$^a$ |
| **(a1)** | 80.37 ± 0.14$^a$ | 87.08 ± 0.26$^b$ | 89.80 ± 0.73$^b$ | 9.44 | 1.56 ± 0.20$^b$ |
| **(a2)** | 77.38 ± 1.02$^b$ | 85.97 ± 0.26$^a$ | 90.99 ± 0.44$^{ab}$ | 13.61 | 1.43 ± 0.38$^b$ |
| **(a3)** | 80.68 ± 0.75$^a$ | 84.43 ± 0.24$^a$ | 88.03 ± 0.34$^c$ | 7.34 | 0.15 ± 0.02$^c$ |
| **(b)** | 72.39 ± 0.22$^b$ | 75.71 ± 0.12$^a$ | 79.55 ± 0.12$^c$ | 7.16 | 15.13 ± 0.15$^a$ |
| **(b1)** | 66.78 ± 0.14$^c$ | 75.43 ± 0.25$^a$ | 81.10 ± 1.02$^b$ | 14.32 | 2.27 ± 0.15$^b$ |
| **(b2)** | 64.66 ± 0.92$^d$ | 73.77 ± 0.73$^b$ | 76.06 ± 0.28$^d$ | 11.40 | 0.98 ± 0.10$^c$ |
| **(b3)** | 76.58 ± 0.43$^a$ | 74.90 ± 0.51$^c$ | 87.50 ± 0.50$^a$ | 10.92 | 0.51 ± 0.12$^c$ |

(*) To inicial temperature, Tp peak temperature, Tc conclusion temperature, ΔHgel gelatinization enthalpy. Values that have the same letter, from the same sample type, don't present significative difference by Tukey's Test ($p<0.05$).

The gelatinisation enthalpies of the modified samples of ginger and yam were intensely decreased, as the milling time increased, as related by Loubes and Tolaba (2014). This suggests that the starches may have undergone pregelatinisation. Cavallini and Franco (2010) explain that ball milling can induce a partial gelatinisation, with similar results to that caused by heating. Pregelatinised starches are formed of damaged granules, soluble in room-temperature water, and with a crystalline disorder, because during this process can occur the depolymerisation or fragmentation of starch molecules (BeMiller and Huber, 2015). In addition, this modification can break down the double helices of amylopectin, due to the rupture of the hidrogen bonds, which stabilise the molecules in the starch granule contributing to the decrease of the enhtalpy (Tester, Karkalas and Qi, 2004).

Shi et al. (2015) also verified a decrease in the gelatinisation enthalpy of maize starch, and the onset temperature was shifted to higher temperature. This was related to the destruction of the crystallinity portion of the granules, implying a weaker expansion capacity of the granules during gelatinisation.

Besides of the enthalpy, the gelatinisation temperatures of the two starch sources also showed a tendency to decrease after the milling treatment as well as identified for cassava starches (Huang et al., 2006), which indicates that the amylose chains were affected too. The amylose depolymerisation and amylopectin degradation by breakage of intermolecular bonds can occur in starches exposed to ball-milling, favouring the appearance of disordered amorphous regions which are easily accessible to water, allowing the gelatinisation at lower temperatures (Liu et al., 2017; Martínez-Bustos et al., 2007; Kim and Kim, 2014).

Loubes and Tolaba (2014) observed an increase in the water absorption capacity, dilatation and solubility of the ball-milled rice flour, and reported that the rate and the time of milling had a negative effect on the temperatures and enthalpy of gelatinisation.

Due to the decrease of crystallinity and the new chain arrangements acquired by the starches exposed to the mechanical forces, as well as the partial gelatinisation of these polysaccharides, Liu et al. (2011) have suggested the use of these as stabilizers, adsorbents, food additives and moisture retention agents.

### *3.1.2. Simultaneous Thermogravimetry and Differential Thermal Analysis (TG-DTA)*

The TG-DTA curves are exposed in Figure 2, for ginger and yam starches. According to Ionashiro, Caires and Gomes (2015), Thermogravimetry (TG) shows the mass loss of sample and differential thermal analysis (DTA) can monitor the heat effects associated with physicochemical changes in the sample. Phase transitions, dehydrations, and reductions produce endothermic effects, whereas crystallisations, oxidations and some decomposition reactions produce exothermic effects.

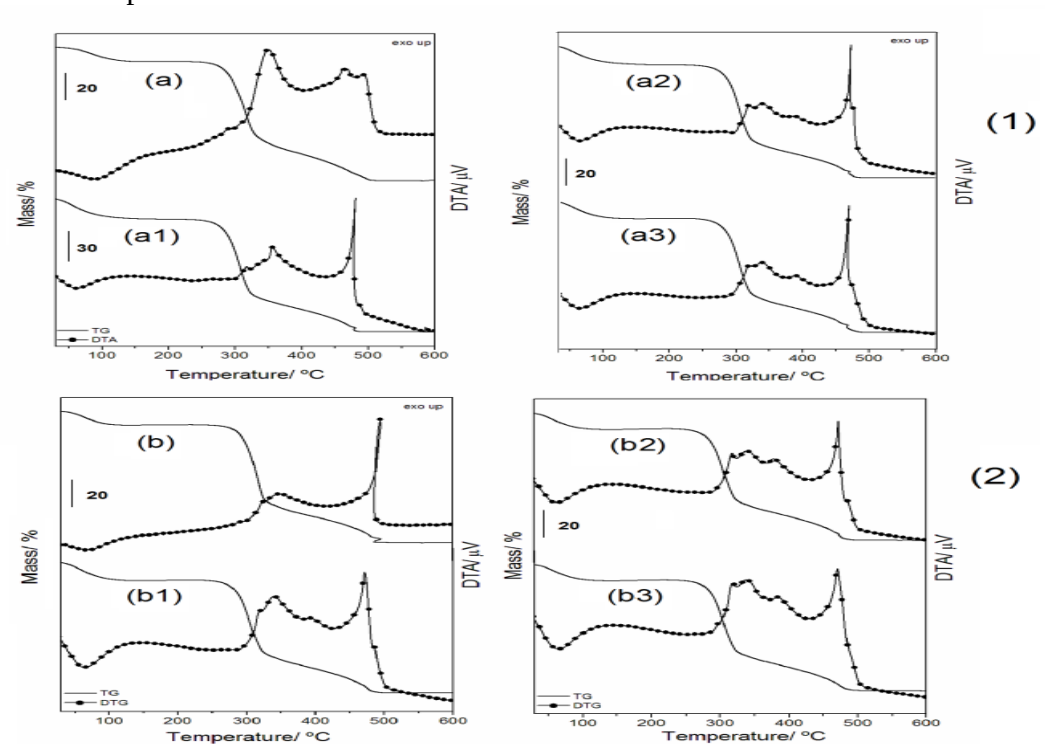


**Figure 2**. TG-DTA curves of the native and modified starches from ginger (1) and white yam (2).

It was noted that the thermal decomposition of the samples occurred in three consecutive mass losses, where the first one was related to sample dehydration, comprising temperatures between 30 – 157 ºC for ginger starches, and 30-140 ºC for white yam starch samples. A stability plateau can be observed after water loss, which increased after milling process. The white yam starch that remained in the ball mill for 40 min presented a higher stability in relation to the native sample, with increased of 11 ºC, and for ginger starch, after 20 min of milling, the stability increased 8 ºC.

The second step referred to the decomposition of the organic matter of the samples, occurring between 247-375 ºC for the samples of ginger starch and between 245-372 ºC for the white yam starch samples.

At temperatures above 300 ºC the starch depolymerisation occurs, and in oxygen atmosphere this decomposition is exothermic (Cordoba, Bet and Schnitzler, 2015). According to Aggarwall et al. (1997) in some cases, the breakdown of amylopectin binds contributes to the decomposition of starch granules at high temperatures.

The third mass loss was attributed to the oxidation of organic matter occurring

between 338-512 ºC and 341-519 ºC for ginger and white yam starch samples, respectively.

The ash content of each sample was: (a) 0.4; (a1) 0.7; (a2) 0.5; (a3) 0.5; (b) 0.8; (b1) 0.7; (b2) 0.2; (b3) 0.2 %, respactively.

**Table 2**. Results of the thermogravimetry-differential thermal analysis for the untreated (a) and modified starches from ginger (a1, a2 and a3); and untreated white yam (b) and modified white yam (b1, b2 and b3).

| | **Steps** | **TG Results** | | **DTA results** |
|---|---|---|---|---|
| | | $\Delta_m$(%) | ΔT (ºC) | $T_p$ (ºC) |
| **a** | 1st | 10.9 | 30-149 | 87.58 |
| | Stb. | - | 149-247 | - |
| | 2nd | 67.5 | 247-375 | 350.2 |
| | 3rd | 21.2 | 375-512 | 460.4 |
| **a1** | 1st | 13.3 | 30-157 | 61.9 |
| | Stb. | - | 157-249 | - |
| | 2nd | 62.2 | 249-357 | 239.7 |
| | 3rd | 23.8 | 357-506 | 480.9 |
| **a2** | 1st | 14.1 | 30-145 | 62.3 |
| | Stb. | - | 145-255 | - |
| | 2nd | 60.9 | 255-339 | 290.7 |
| | 3rd | 24.5 | 339-508 | 470.2 |
| **a3** | 1st | 12.8 | 30-131 | 62.7 |
| | Stb. | - | 131-247 | - |
| | 2nd | 60.8 | 247-338 | 240.1 |
| | 3rd | 25.9 | 338-512 | 468.4 |
| **b** | 1st | 11.6 | 30-135 | 67.5 |
| | Stb. | - | 135-245 | - |
| | 2nd | 65.5 | 245-372 | 347.3 |
| | 3rd | 22.1 | 372-519 | 493.9 |
| **b1** | 1st | 13.9 | 30-136 | 64.2 |
| | Stb. | - | 136-252 | - |
| | 2nd | 59.6 | 252-341 | 276.2 |
| | 3rd | 25.8 | 341-517 | 471.4 |
| **b2** | 1st | 12.5 | 30-140 | 62.3 |
| | Stb. | - | 140-254 | - |
| | 2nd | 62.7 | 254-341 | 275.6 |
| | 3rd | 24.6 | 341-524 | 472.4 |
| **b3** | 1st | 12.1 | 30-137 | 64.82 |
| | Stb. | - | 137-256 | - |
| | 2nd | 61.6 | 256-341 | 252.9 |
| | 3rd | 26.1 | 341-511 | 470.8 |

Δm, mass loss (%); ΔT, temperature range (°C); Tp, peak temperature (°C), Stb., stability.

### 3.2. Morphological results

The micro-images of the native and grinded granules of ginger and yam starches are shown in Figure 3. Native ginger starch presented oval shape with some spherical granules, without evidence of cracks, as also obtained by Zhang et al. (2012). Native yam starch granules exhibited an oval shape with some triangular granules and a smooth surface, as reported by Hornung et al. (2017).

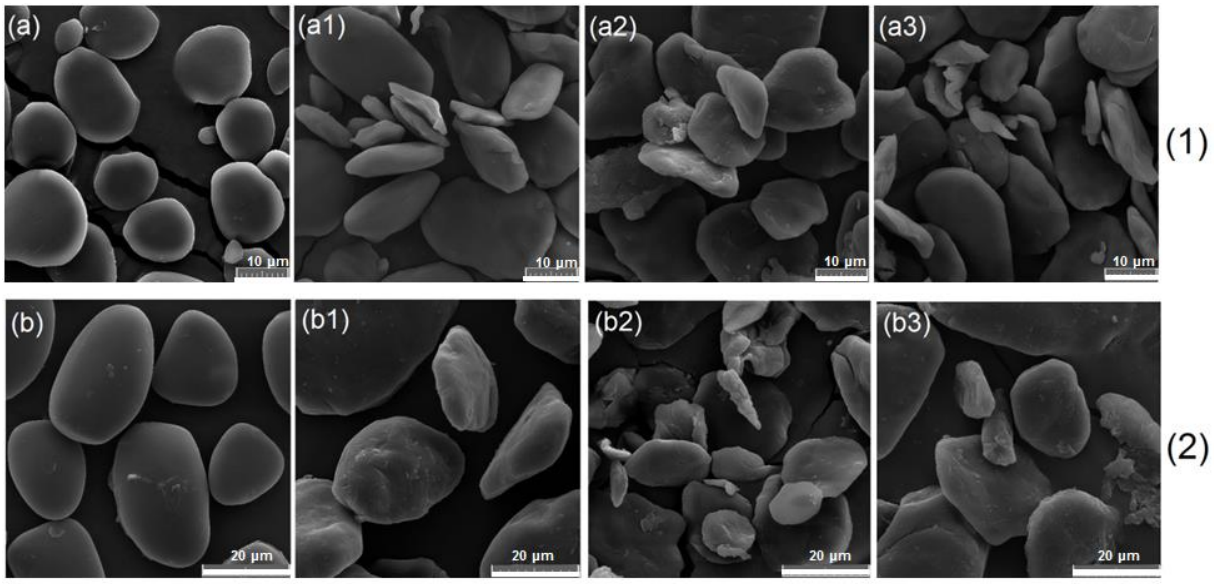


**Figure 3**. Micro-images of: (1) native and modified ginger starch granules, and (2) native and modified white yam starch granules.

According to Diop et al. (2012), during the milling process the starch granules are peeled layer by layer, transforming into anomalous and small granules. There was a physical degradation in the grinded starches, which were losing their shape as the milling time increased. These deformations can be caused by the friction between the starch granules, water molecules, milling balls and the wall of the cylinder, resulting in the production of heat, which, in addition to mechanical energy, contributed to change the shape and the properties of the starch (Martínez-Bustos et al., 2007).

Ball-milling process can cause cracks on the surface of the granules, which favour the passage of water, leading to the formation of fragments that can swell in cold water. Any starch subjected to the milling may contain damaged and fragmented granules (BeMiller and Huber, 2015).

The cracks that appear after the modification facilitate the diffusion and increase the susceptibility to hydrolysis, since the fragments present a larger surface area, as discussed by He et al. (2014).

Liu et al (2011) identified that after 1 h of milling, the surface of the corn starch granules became rough, losing smoothness and flatness. They observed grooves, cracks and a few small fragments of granules with 2 h of treatment, maintaining the integrity in the granule periphery. And finally, after 3 h of milling, the granules were divided into smaller pieces and agglomerates. In the present study it were not identified agglomerates, maybe due the lower milling time employed.

Su et al. (2016) observed after 6 h of milling, the appearance of rough and exfoliated surfaces, due to the removal of small pieces of the external layer of corn starch granules, by the action of the pressure and friction of the balls

The average lengths (Table 3) of the ball-milled granules decreased compared to the

native starches, as observed by Ren et al. (2010) for cassava starch. In this treatment, starch granules are subjected to various forces such as compression, impact and shear, which contributes to damage and decrease the size of the granules (Huang et al., 2007).

For the ginger starch, the average size of the granules decreased proportional to the time spent on the ball mill. The same happened with the white yam starch, except after 40 min.

### 3.3. Structural parameters

#### *3.3.1. Powder diffraction and relative crystallinity*

The X-ray diffractograms of the native and modified ginger and white yam starches are shown in Figure 4. It was identified an A-type diffraction pattern for native ginger starch as reported by Zhang et al (2009) for yellow ginger starch. And the native white yam starch was classified as B-type diffraction pattern, as also obtained by Hornung et al (2016).

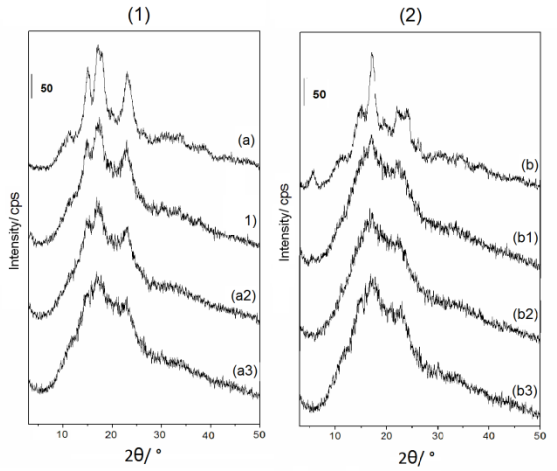


**Figure 4**: X-ray diffractograms of: (1) native and modified ginger starch granules, and (2) native and modified white yam starch granules.

Greater exposure of the granules to the grinding caused a decrease in the intensity of each peak and in the relative crystallinity of the samples (Table 3), as discussed by Anzai et al. (2011). Shi et al. (2015), found that ball-milled corn starch maintained A-type pattern, but had a drastically decrease in its crystallinity.

Ground samples lose their crystalline structure due to the high energies and mechanical force employed during ball milling. As the amorphous regions tend to increase, the degree of relative crystallinity decreases, since a change occurs in the amylopectin and amylose arrangements (Roa et al., 2014).

**Table 3**. Results of relative crystallinity by XRD and average length of the starch granules, by SEM.

| Samples | Relative crystallinity (%) | Average length (µm) |
|---|---|---|
| a | $30.09 \pm 0.87^{a}$ | $20.47 \pm 1.13^{a}$ |
| a1 | $23.18 \pm 0.44^{b}$ | $14.12 \pm 0.97^{b}$ |
| a2 | $20.08 \pm 0.68^{c}$ | $13.68 \pm 1.15^{bc}$ |
| a3 | $19.50 \pm 0.58^{c}$ | $11.99 \pm 0.75^{c}$ |
| b | $27.91 \pm 0.90^{a}$ | $30.01 \pm 2.49^{a}$ |
| b1 | $17.54 \pm 0.22^{b}$ | $15.29 \pm 1.04^{c}$ |
| b2 | $13.31 \pm 0.28^{c}$ | $10.55 \pm 1.20^{d}$ |
| b3 | $13.69 \pm 0.40^{c}$ | $18.32 \pm 0.69^{b}$ |

(*) Values that have the same letter, from the same sample type, don't present significant difference by Tukey's Test ($p<0,05$).

Starch is considered a semi-crystalline polymer due to lateral branching of amylopectin, which forms a double helix structure. However the hydrogen bonds which maintain stable this structure can be broken during grinding, exposing the hydroxyls and giving a more amorphous character (Anzai et al., 2011). It is probable

that this occurred in this study, observing the fall in the relative crystallinity, associated with the decrease in the gelatinisation enthalpy, evidenced by DSC.

Roa et al. (2014) studied an enriched fraction of amaranth starch (*Amaranthus cruentus*) modified with ball mill and observed the destruction of the ordered structure of the starch, proportional to the degree of energy used. There was a decrease in crystallinity with increasing amorphous regions. As a consequence, a higher capacity of water absorption and cold solubility of the flour was obtained.

### *3.3.2. Fourier Transform Infrared Spectroscopy (FT-IR)*

Figures 5 and 6 show the Fourier transformed infrared spectra of ginger and yam starches, respectively.

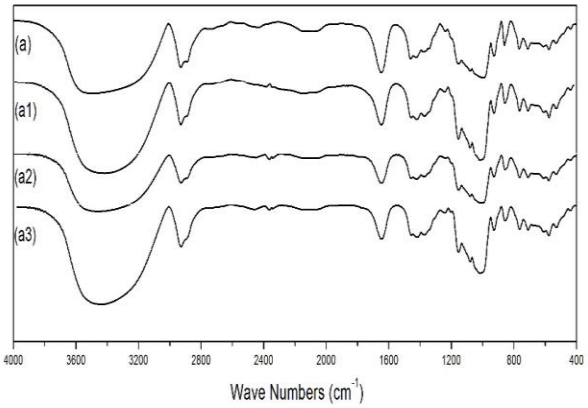


**Figure 5**. Fourier transformed infrared spectra for native and modified ginger starch granules.

Bands commonly observed in starch spectra correspond to the wavenumbers of 850, 996, 1014, 1039, 1145 and 1075 $cm^{-1}$. They are related to bending and stretching of C-O-C groups and C-O, C-O-H of the glycosidic bonds, according to Roa et al., (2014). In this study, these bands can be visualised in the spectra of ginger and yam starches, which underwent slightly changes caused by ball milling treatment, as observed for ball-milled maize starch (Liu et al., 2011).

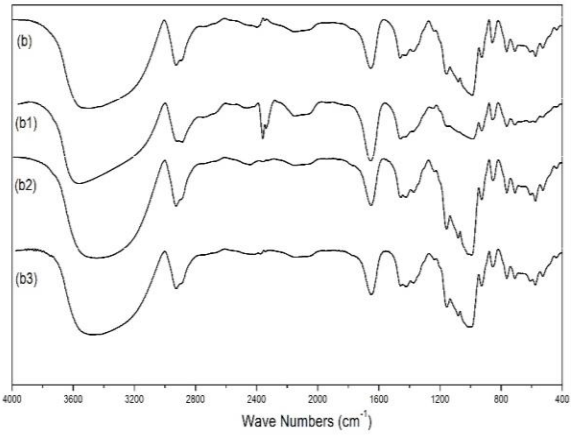


**Figure 6**. Fourier transformed infrared spectra for native and modified white yam starch granules.

Changes in the starch bonds are expected after treatment with ball mill, as it may cause the depolymerisation of the starch chains, reorganizing its structure (He et al., 2014).

The band at 3500 $cm^{-1}$ was attributed to the stretching vibration of OH- molecules (complex stretching vibration associated with free intra- and inter-molecular bound hydroxyl groups), and the band closest to 3000 $cm^{-1}$ was related to C-H bond stretching (Chang et al., 2014).

The band at 2400 $cm^{-1}$, observed in the spectra of b1 sample was attributed to the stretching of $CO_2$ molecule, associated in the preparation of the sample pellet.

Bands at 1480 $cm^{-1}$ were related to stretching vibration of C-N bond (Wang and Xie, 2010). This band may point to a presence of proteins in the ginger and yam starches.

## 4. Conclusions

From the study of ball milling applied to ginger and white yam starches, it was possible to observe changes on the thermal, morphological and structural properties of the granules.

The gelatinisation enthalpy and transition temperatures of the modified samples, both ginger and white yam, suffered a decrease compared with the native starches, mainly at higher milling times.

By differential thermal analysis it was observed three mass losses. For the ginger starch, it was identified that the ball-milled samples for 20 and 40 minutes had an increase in their stability period. As for the white yam starch, it was observed that the stability period of the modified samples increased in proportion to the exposition time in the ball milling process.

It was observed a substantial change in the samples morphology. The granules lost their surface smoothness, had a decrease in the size and showed fissures and roughness after the modification.

The relative crystallinity of the ground starches decreased with the increase of the milling time.

Slight changes occurred in the infrared spectra, especially in bands smaller than 1000 $cm^{-1}$, corresponding to the glycosidic bonds.

It was concluded, therefore, that the time utilised on this paper was enough to modify the studied starches. Also, 20 minutes modification caused as much alteration on the starches properties as the 40 minutes one, possibility a save in time and energy.

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# STABILITY OF VITAMIN C AND ß-CAROTENE DURING PROCESSING OF PAPAYA GUAVA FRUIT LEATHER

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**ABSTRACT**

Papayas and guavas are underutilized nutrient rich tropical fruits widely grown in Rwanda. It was envisaged that processing them into novel fruit leather would enhance their consumption. Hence, fruit leather was processed and their acceptability assessed after processing. Papaya (Mexican and Hawaiian varieties) and guava in 60:40 ratio with sugar (45 %) and citric acid (1 %) were blended; dried mechanically and subjected to chemical and sensory analysis. Moisture content was high in fresh fruits both in papayas (89.2%), guavas (84.2%); and decreased (16.4%) upon leather processing. Total Soluble Solids increased from 8.8$^0$Bx to 54.6 $^0$Bx in Mexican papaya and 8.6 $^0$Bx to 53 $^0$Bx for Hawaiian papaya. Total reducing sugar doubled from approximately 8% - 9% in fresh fruits to 20% in the processed fruit leather. Carbohydrate increased by nine times in processed fruit leather when compare to fresh sample. Protein content in fresh papaya (0.55%) and guava (1.3%) increased to 3.35% in papaya leather. Fat in fresh fruits were low (0.2% - 0.5 %) but increased to 1.2% in leather. Vitamin C in both fresh fruits of papaya (20mg / 100g), irrespective of the variety, and guava (24.8 mg/ 100g) were nearly the same. Processing decreased it to 13.3 mg /100g and 13.5 mg /100g in the Mexican and Hawaiian variety respectively; titratable acidity increased while pH decreased (3.8). ß-carotene content in fresh papaya was quite less (0.120 mg / 100g) irrespective of its variety; while in guava it was 3.22 mg / 100g. Thermal processing brought about degradation of carotene content. There was no statistical difference (p-value>0.05; p-value>0.01) between papaya varieties.

## 1.Introduction

Fruits and vegetables form an integral part of the dietary habits of human beings. They are fundamental sources of vitamins and minerals (Kader, 2001). Vitamins are recognized to be potent compounds that perform vital tasks in the body such as growth, reproduction and maintenance of life. Since vitamins and mineral play a significant role in the body's metabolic activities, their deficiencies causes deficiency diseases; however, prolonged lack of micronutrients leads to hidden hunger. Fruits usually contain large amount of water and therefore are highly perishable. Hence, they are processed into various shelf stable forms adopting drying, freezing, concentration and fermentation technologies, not only to prevent their postharvest loss but also to enhance their acceptability. Rwanda being a small land locked country in East African region is also confronted with the challenges of micronutrient deficiency diseases. In Rwanda, 38% of children; it is high in rural than in urban and high among boys than girls under 5 years of age (World Bank, 2018). Anemia among preschool aged children and pregnant women were 42% and 11% respectively (WHO, 2008).

Rwandan diets are dominated by plant based foods such as cassava, maize, beans, potatoes, bananas and are very low in fat (≈8%) (Ho & McLean, 2011). One of the key actions recommended by World Bank for Rwanda is to improve dietary diversity through promoting home production of diversity of foods (World Bank, 2009). However, several collective efforts have been taken by the Ministries of Agriculture and Health to overcome them. According to the 2010/11 Integrated Household Living Conditions Survey in Rwanda, there has been a dramatic change in the production of cultivating only staple food crops at household levels to that of cultivating a combination of staple and wide range of fruits and vegetables (NISR, 2011).The widely cultivated fruits and vegetables include mangoes, papaya, avocadoes, pineapples, guava, oranges, mandarins, lemons, grapefruits, passion fruits, strawberries, fresh beans, green beans, tomatoes, onions, garlic, peppers, squash, zucchini, eggplants, carrots, leeks, lettuce, parsley and mushrooms. Such efforts have improved the dietary quality of meals but the postharvest loss is still high. The best approach is to adopt processing techniques and technologies to conserve and utilize them which otherwise would be easily jeopardized.

Papaya, *Carica papaya* L., is one such fruit that has not been exploited much to be processed. Of recent, they are widely cultivated and available in many of the homesteads. It is a large herb and member of the small family *Caricaceae* (Morton, 1987). They are major tropical and sub-tropical fruit crop in the world. Papayas are climacteric fruits and they undergo a series of desirable biochemical changes after harvesting. It has been documented that several tropical fruits are rich in antioxidants such as polyphenols, carotenoids and vitamins (Corral-Aguayo, Yahia, Carrillo-Lopez, & Gonzalez-Aguilar, 2008) of which papaya is one. ß carotene is an important pre cursor of vitamin A and contributes to the attractive color of the fruit. Papayas also contain vitamin C, B, potassium, magnesium, folic acid, fiber, low in calories, but aids in digestion (Ian, 2004); the lack of tartness is due to the low acidity in the fruit (Ursell, 2000) . Therefore they are vital for human nutrition and health and promote healthy cardiovascular system, protection against colon cancer (Nimmanpipug, Therdthai, & Dhamvithee, 2013) and would therefore be beneficial in the prevention of diabetic heart diseases (Fernandes, Rodrigues.S, & Oliveira, 2006). One way papayas are usually consumed is as fresh fruits but also as processed products. They are commonly made into sauce for shortcake or ice cream sundaes, added to ice creams just before freezing, used as pie filling, pickled, or preserved as marmalade or jam (Morton, 1987). They are also used as osmotically dehydrated preserves used as snacks or as fruit toppings in the bakery and confectionery industries (Nimmanpipug, Therdthai, & Dhamvithee, 2013). Mature green papayas are also consumed as a vegetable in several parts of India, Thailand and Vietnam (Morton, 1987). Furthermore, they can be consumed as restructured fruit in the form of leather. Consuming papayas as processed products helps to utilize and minimize the postharvest loss experienced through mechanical injury (Chonhenchob & Singh, 2005) during transportation and distribution. Guava is a fruit with pleasant sour-sweet taste and good source of vitamin C (212mg/100g) and dietary fiber (5.2g / 100g) (Gopalan, Rama Sastry, & Balasabramanium, 2009). They contain almost five times as much vitamin C as oranges viz., 30mg /100g in the fruit and 64mg /100ml in the juice (Gopalan, Rama Sastry, & Balasabramanium, 2009). National Institute of Nutrition, Hyderabad, India found antioxidant concentrations of 500mg / 100 g in guavas (Dean, 2011). Guavas (*Pisidium guajava)* are cultivated, in Rwanda, as a home garden crop and are considered as a low-priced food for poor but are inexpensive source of nutrients compared to other fruits grown in Rwanda.

Fruit leathers, otherwise called as fruit bars or fruit slabs are considered to be a nutrient

dense, convenient, economically value added substitute for natural fruits and an excellent alternative for calorie dense snacks and very popular in United States of America (Artthey & Ashurt, 1984; Huang & Hsieh, 2005) than in Africa. It can also be used as nutrient dense food for people on hikes or mountain treks, astronauts on space mission. Fruit leathers can be made from any type of fruits including guava, durian, jackfruit, mango, pears, papayas, pineapples, banana, sweet potato and several other fruits (Phimpharian, et al., 2011; Chowdhury, Bala, & Haque, 2011; Huang & Hsieh, 2006; Babalola, Ashaye, Babalola, & Aina, 2002; Irwandi, Man, Yusof, Jinap, & Sugisawa, 1998; Wandi & Man, 1996; Chan & Cavaletto, 1978; Chan & Cavaletto, 1982). Fruit leathers are made by mechanical or solar drying of the fruit puree and combining other added ingredients such as sugar to enhance the sweetness, citric acid to increase the acidity and chopped nuts, coconut or spices to vary the taste and flavor (Huang & Hsieh, 2005). Blending of fruit purees to make fruit leathers enhances the nutritional quality and prevents postharvest loss. However, very little has been reported on the formulation of blended fruits in the development of fruit leathers. Both papayas and guavas are underutilized in Rwanda, except for fresh consumption, when compared to other tropical fruits such as pineapples, passion fruits, bananas, tree tomatoes, to name a few. Adding value to papaya and guava as they are less appreciated fruits and creation of new products from papaya and guava will help consumers to obtain vitamins when it is processed as leather. This will also complement the in-home fortification approach adopted in Rwanda to enhance the consumption of micronutrients and also balance school foods of the children. Hence blending papaya with guava would not only enhance the nutritional quality but also harmonize and complement the final product in its nutritional quality, sensory attributes and consumer preferences. Therefore the study aimed to process fruit leather from guava blended papaya and comparison of their chemical properties and consumer preference between the Mexican and Hawaiian varieties of papayas.

## 2. Materials and methods

### 2.1. Materials

#### *2.2.1. Sample preparation and storage*

Fresh papaya (Mexican and Hawaiian) and guava fruits were purchased from the local market and brought to the food processing laboratory and refrigerated until processing. All the fruits selected were ripe and free from bruising, to protect the color and flavor of the final leather product. The fruits were then washed in potable chlorinated water, peeled and their seeds removed. The fruits were hygienically cut into pieces and processed into homogenous puree individually in a stainless steel Waring commercial blender (Model no: HGBSSSS6, Torrington, Connecticut, USA) for 3 minutes; consecutively three times at 5 minutes interval. The liquidized fresh fruit puree (Papaya Haw, Papaya Mex & Guava) were strained or sieved to remove fibers to obtain smooth puree. The papaya – guava leather formulation was in the ratio of 60:40 for both the Mexican and the Hawaiian variety. Sugar and citric acid was added at the rate of 45% and 1% respectively. They were thermally processed at $90^0$ C in a stainless steel double pan to prevent overheating. The fruit puree was poured in a thin layer (3-6mm thick) on stainless steel trays lined with greaseproof paper. The trays with the sample puree were left on the bench top at ambient temperature of 25 $^0$ C for a minute to allow the even distribution of the sample puree. They were then placed in a mechanical dryer ( UNITEMP Drying cabinet, LTE Scientific Ltd., Greenfield, Odham, UK) and dehydrated at 60 $^0$C for 8 h. They were hygienically cut, wrapped and stored at ambient temperature in air tight dry containers.

## 2.2. Chemical analysis

### *2.2.1. Moisture content*

It was determined by using oven drying method (Ranganna, 2001) where the food sample of 5g was heated at 110 $^0$ C for 1hr and then weighed for weight loss. The experiment was continued until there was no variation in the consecutive weighing. Finally moisture content was expressed in percentage.

### *2.2.2. Sugar content*

Lane-Eynon method was used to determine the sugar content. The carbohydrate solution to be analyzed was taken in a burette and added to a flask containing known amount of boiling copper sulphate solution and methyl blue indicator. Absorbance was measured at 490nm and the amount of sugar was determined by reference standard curve (Ranganna, 2001).

### *2.2.3. Protein content*

Micro Kjeldahl method was adopted to determine protein content (Ranganna, 2001). The samples were prepared by blending manually in a mortar and pestle. Potassium sulphate (1g) was transferred to 250ml digestion tube. They were digested during 1h at 400-450$^0$C, therefore there was distillation and titration with 0.1N hydrochloric acid and sulphuric acid solution and the end point was shown by a light color. The crude protein was expressed in percentage.

### *2.2.4. Fat content*

Soxhlet method after 8-16hrs extraction was followed. The sample was digested by heating it for 1h in the presence of 3N-HCl then the fat content was expressed in percentage (Horwitz & Latimer, 2005).

### *2.2.5. Ash*

Ash content was determined using a muffle furnace (LF3, Vecstar Ltd, Furnace Division, Chesterfield, UK). 5g of the sample was accurately weighed into a crucible. The muffle furnace was heated to 550$^0$ to 600$^0$ C and the sample was incinerated for 6 to 8 hrs. It was cooled in desiccators and then weighed to obtain the weight of the ash. The experiment was repeated three times.

### *2.2.6. Ascorbic acid content*

It was determined by using 2, 6 dichloro - phenolindophenol visual titration method (Ranganna, 2001). This method was based on the reduction of 2, 6 dichlorophenolindo - phenol by acid and those based on the reaction of dehydroascorbic acid with 2,4 dinitrophenyl hydrazine. 5g of papaya leather for both treatments were weighed and subjected to final ascorbic acid content determination and standardization of indophenols solution.

### *2.2.7. Total reducing sugar*

Lane-Eynon procedure was followed as outlined in Official Method of Analysis of AOAC International (Horwitz & Latimer, 2005) where the Fehling solution was standardized and the preliminary titration and final titration was done. Then total reducing sugar was expressed in percentage.

### *2.2.8.Total Soluble Solids (TSS)*

Refractometer (model number: digit 090; 0- 42$^0$Bx and 42$^0$ – 71$^0$Bx- precision 0.2$^0$Bx; CETI, Medline, Oxfordshire, UK) was used to determine the total soluble solids.

### *2.2.9. pH*

Bench model pH / mV meter (HANNA Instruments, model no: HI-122, Romania) was used.

### *2.2.10. ß carotene*

Spectrophotometer Camspec (model: M 501single beam scanning UV visible spectrophotometer) was used to determine the ß carotene content (Horwitz & Latimer, 2005).

## 2.3. Sensory analysis

Descriptive analysis was used to define the sensory properties of the newly developed papaya-guava fruit leather. The protocol and procedures employed in the research study

were reviewed and approved by the review committee of the Directorate of Research and Publication office, CAVM. The study followed ethical standards and procedures laid down for the sensory analysis of newly developed food products. A total of thirty final year fellow graduate students between the ages of 21 and 25 years comprising of both males and females were recruited for this analysis. All panelists volunteered to participate and were encouraged to test the products from two varieties (Hawaiian and Mexican) of papaya, blended with guava, where a list of parameters was used to identify the products and to make a comparison of both varieties from papaya. Sensory quality parameters measured included: overall acceptability, taste, flavor, texture and color. Panelists were also instructed to comment in detail regarding the above parameters what in particular they have liked or disliked about these products.

**2.4. Statistical analysis:**

All laboratory analysis were carried out in triplicates. The mean and standard deviation was calculated using MS Excel for all the chemical parameters analyzed and sensory attributes were investigated by using differences in Analysis of variance (ANOVA) at 5% and 1% level of significance.

**3.Results and discussions**

The fresh fruit pulp and developed papaya – guava fruit leather products were analyzed for their carbohydrates, protein, fat, ash, moisture content, brix, reducing sugar pH, TTA, vitamin C and β-carotene. Three replicates were done (Table 1).

**3.1. Chemical analysis of fresh and processed samples**

***3.1.1. Carbohydrates, TSS and total reducing sugar content***

The carbohydrate content in fresh papayas irrespective of the varieties ranged from 8.4% to 8.6%; guavas had nearly double the amount (14%). The addition of sugar and the concentration of the fruit puree mixture through thermal processing increased the concentration of sugar that reflected an increase in TSS from $8.8^0$Bx to $54.6^0$Bx in the Mexican papaya and $8.6^0$Bx to $53^0$Bx for the Hawaiian papaya; which indicated that no significant difference existed in TSS between the varieties of papaya (Fig 1). The total reducing sugars in the fresh papaya and guava fruits ranged from 8% to 9% whereas the leather irrespective of the variety of papayas had 20% of reducing sugar. Carbohydrates are very prominent constituents of plants that serve not only as a source of available energy but also as reserve food and as structural materials. They are one of the main groups of food substances other than proteins, and fats to be synthesized in the plant from simple organic substances. Sugars are an important component in fruits; total sugars vary from 3% to 18% and consist of a mixture of sucrose, fructose and glucose (Swaminathan, 1988). Sugars can act as reducing agents and these sugars contain aldehyde as the functional group. Reducing sugars are the free hexose and pentose content of foods and are generally reported only as "total reducing sugars" (Lee, Shallenberger, & Vittum, 1970) which is lower than the carbohydrate content in the papaya leather which had a score of 76.0% as shown in table1. It is known that papaya leather contains high carbohydrates than fresh papaya.

***3.1.2. Protein content***

The protein content in fresh papaya was 0.55% while in guava it was 1.3%; it increased to 3.35% in final product of papaya guava leather. In dried or dehydrated form, nutrients increase and this resulted in concentrated form of protein. Protein content in fruit is very minimal.

***3.1.3. Fat***

The fat in the fresh fruits were low and ranged from 0.2% to 0.5 % while in the leather it increased to1.07±0.01 to 1.2±0.00%. Fruits

are poor sources of fats and range from 0.1% to 0.5% (Desrosier & Desrosier, 2006).

**Table 1.** Nutrient content in fresh and processed papaya, guava and its blend

| Nutrients | Fresh Pulp | | | Blended & Processed Leather | |
|---|---|---|---|---|---|
| | **Papaya (Mex. var)** | **Papaya (Haw. var)** | **Guava fruit** | **Papaya (Mex. Var) / guava** | **Papaya (Haw. Var) / guava** |
| **Carbohydrate (%)** | 8.67±0.01 | 8.40±0.03 | 14.00 ±0.03 | 75.53 ±0.89 | 71.87±0.25 |
| **Total reducing sugars (%)** | 8.0±0.09 | 8.2±0.03 | 9.0±0.06 | 20±0.00 | 20±0.2 |
| **Total Soluble Solids (TSS $^0$Bx)** | 8.8±0.06 | 8.6±0.0 | 9.8±0.00 | 54.6±0.2 | 53±0.00 |
| **Fat (%)** | 0.2±0.00 | 0.27±0.01 | 0.53±0.00 | 1.2±0.00 | 1.07±0.01 |
| **Protein (%)** | 0.6±0.00 | 0.53±0.01 | 1.27±0.01 | 3.40±0.00 | 3.27±0.01 |
| **Moisture content (%)** | 89.27±0.03 | 89.23±0.00 | 84.20±2.16 | 16.47±0.44 | 16.43±0.58 |

**Source:** Primary data; **Note:** Haw. var – Hawaiian variety; Mex. var – Mexican variety

### *3.1.4. Moisture*

Fruits are naturally rich in water content (table 1). The moisture content was higher in fresh papayas (89.2%) and it decreased to 16.4% in papaya-guava (84.2%) and the moisture content of papaya – guava leather. Dried and dehydrated foods are highly concentrated, less costly to produce and require minimum storage needs (Desrosier & Desrosier, 2006). Besides thermal processing, dehydration was one of the unit operations used to process fresh guava - papaya fruit leather. Dehydration permits food preservation by reducing the water activity level that does not support microbial growth whilst thermal processing inactivates microorganism and deteriorative enzymes to preserve food. This concept of hurdle technology relies on two or more factors or hurdles (Leistnera & Gorris, 1995) in order to ensure that a given food product remains stable. When free water content is reduced, the osmotic pressure is increased and it facilitates the control of microbial growth in such a system; and moisture content is one of the water related criteria used to study food stability (Salguero, Gomez, & Carmona, 1993).

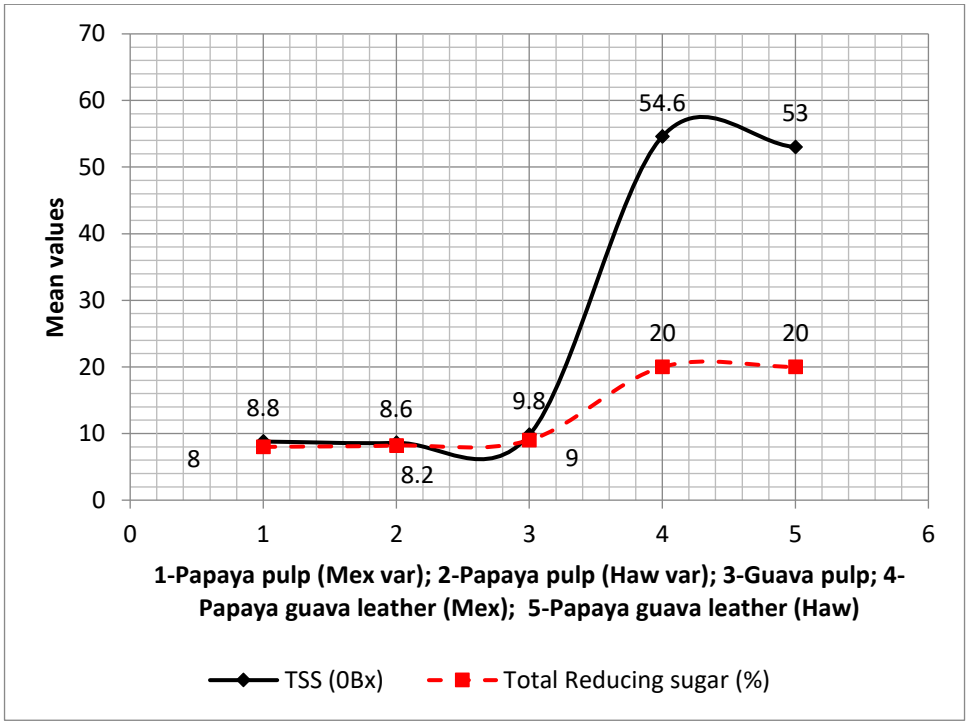
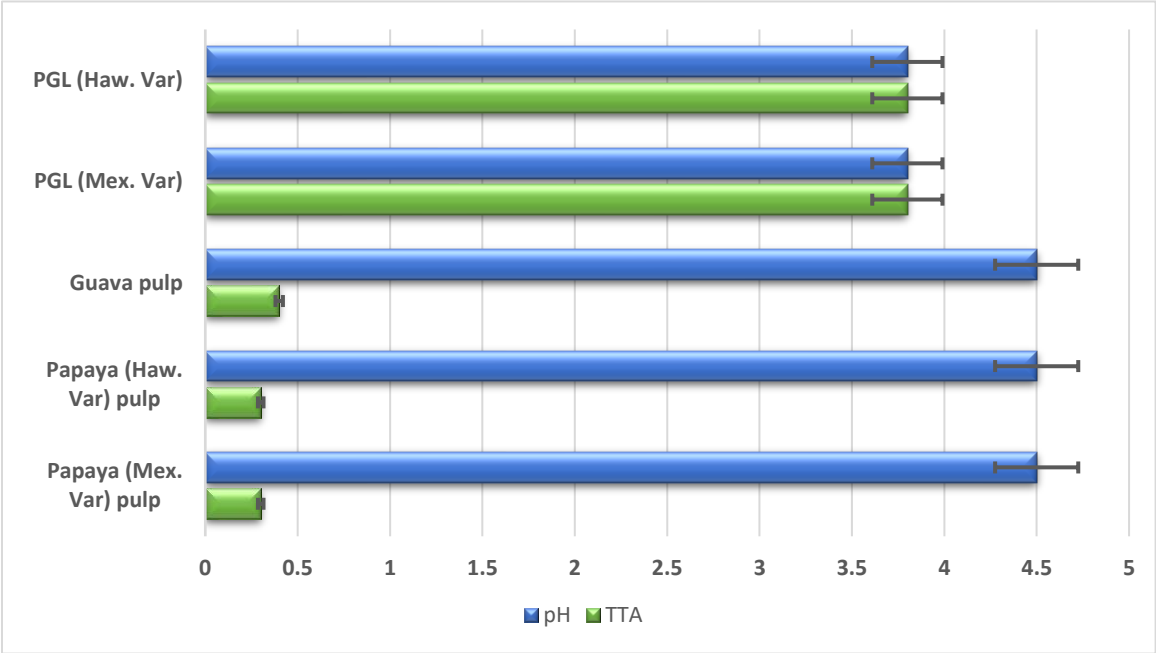
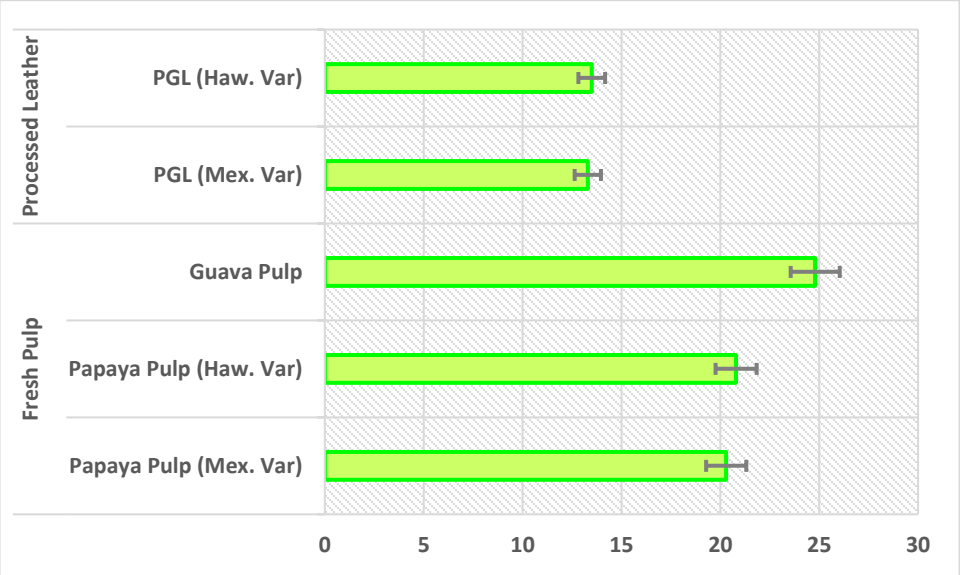



**Figure 1.** TSS and Total Reducing sugar content in fresh fruits and the processed papaya guava leather




**Note: PGL – Papaya Guava Leather; Haw. Var – Hawaiian variety; Mex. Var – Mexican variety**

**Figure 2.** TTA and pH content in fresh fruits and the processed papaya guava leather




**Note: PGL – Papaya Guava Leather; Haw. Var – Hawaiian variety; Mex. Var – Mexican variety**

**Figure 3.** Vitamin C content in fresh fruits and the processed papaya guava leather

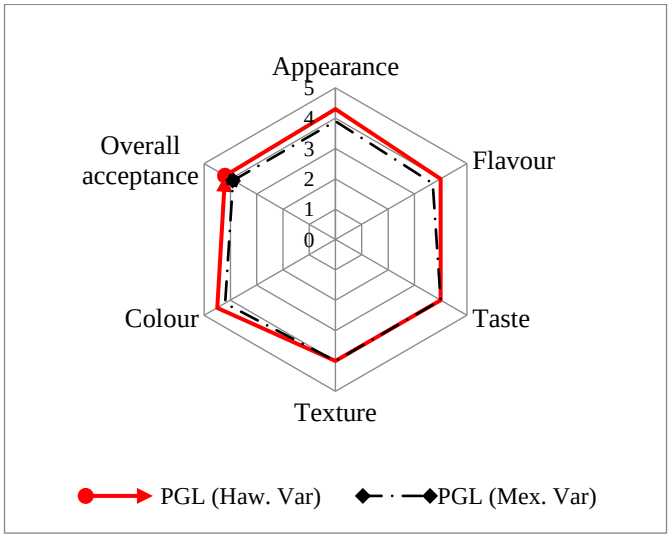



**Figure 4.** Descriptive sensory attributes of fruit leather between two papaya varieties blended with guava

### *3.1.5. TTA and pH*

There are two interrelated concepts in food analysis that deal with acidity; they are pH and titratable acidity. From the analysis it was clear that both varieties of papayas and guava had meagre levels of acidity but their pH was all uniform and fair (4.5). In the papaya-guava leather prepared from both varieties, the titratable acidity increased while the pH decreased and they gave a uniform value of 3.8 (fig 2). However, titratable acidity is a better predictor of acid's impact on flavour than pH (Nielsehn, 2001). The addition of citric acid (1%) increased the titratable acidity and made

the product acceptable; otherwise papaya is a bland fruit. Titratable acidity deals with the measurement of total acid concentration that is contained within a food (Nielsehn, 2001) . Citric and malic acids largely occur in fruits apart from the many other organic acids that occur in them (Desrosier & Desrosier, 2006). Mango leather developed was reported to have low pH of 3.8 that enabled it to be stored and microbiologically safe for a period of six months (Azeredo, Brito, Moreira, Farias, & Bruno, 2006). This pH value correlated with the present study.

### *3.1.6. Vitamin C*

Vitamin C in both the fresh fruits of papaya, irrespective of the variety, and guava were nearly the same; they were 20mg / 100g and 24.8 mg/ 100g in fresh papayas and guavas respectively. On processing they decreased to 13.3 mg /100g and 13.5 mg /100g respectively in the Mexican and Hawaiian variety (Fig 3). This decrease in the vitamin C content could be due to its chemical nature of being sensitive to heat and light. Vitamin C also known as ascorbic acid is derived from simple sugars (Klenner, 1953). It is the most active reducing agent known to occur naturally in living tissues and abundant water soluble antioxidant in the body (Salunkhe & Kadam, 1998). It is important for the growth and maintenance of healthy bones, teeth, gums, ligaments and blood vessels; responsible for the chemicals in the production of neurotransmitters and adrenal gland hormones; involved in the response of the immune system to infection and wound healing Vegetables (Salunkhe & Kadam, 1998) and fruits are good sources of vitamin C and generally citrus fruits are said to be rich source. However, the Indian gooseberry (*Phyllanthus emblica*) has higher (600mg /100g) amount than the citrus fruits. Papaya and guava are excellent sources of vitamin C and contain 57 mg and 15 mg / 100g respectively (Gopalan, Rama Sastry, & Balasabramanium, 2009).

Several studies have indicated that Vitamin C content in fruits and vegetables can be influenced by various production factors such as genotypic differences, pre-harvest climatic conditions and cultural practices, maturity and harvesting methods, and postharvest handling procedures (Nagy, 1980; Lee & Kader, 2000). Ascorbic acid content in the fruits decreases significantly during ripening storage periods (Othman, 2009). Studies on the chemical changes and sensory quality during processing and storage of aseptically "bag-in-box" packaged papaya and guava puree revealed that ascorbic acid loss was 6% during aseptic processing (Cavaletto, 2008). In the present study the loss was quite high (39.24%); which may be due to the open pan thermal processing technique that was adopted. Durian leather was developed and reported to have high amount of ascorbic acid content (21.6 to 26.6 mg / 100g) (Wandi & Man, 1996). The Recommended Dietary Intake (RDI) per day for an adult is 45 mg and 100g of the processed leather can provide 16% of the RDI.

### *3.1.7. ß Carotene*

Colour of fruits and vegetables are very important from the point of view of ultimate quality of the product and eye appeal that enhances consumer acceptability. Chlorophyll, anthoxanthins, anthocyanins and carotenoids are the chief pigments that are present in fruits and vegetables; carotenoids are a group of yellow, orange and orange red fat soluble pigments that are widely distributed in nature. They are highly unsaturated, and are susceptible to isomerization and oxidation during the processing and storage of food. Carotenoid composition in papaya and major carotenoids found in papaya extracts are lycopene and carotenol fatty acid esters of ß-cryptoxanthin and ß-cryptoxanthin-5, 6 epoxide (Cano, de Ancos, & Lobo, 1996). Other xanthophylls detected were ß cryptoxanthin, transzeaxanthin and cryptoflavin. The present study revealed that ß-carotene content in fresh papaya was 0.120±0.00 mg / 100g irrespective of the papaya variety used in the study. This correlated with literature values of 0.0045-.676 mg / 100g (Pamplona, 2003). While in fresh guava, the ß-carotene content was high with a

value of 3.22 mg / 100g when compared to papaya. Guava contains very high lycopene and contains 26 RAE ß-carotene (Percival & Brooke, 2014). The 'Horana red' variety of guava contained 2.0 +/- 0.2 µg/g fresh weight of β-carotene (Chandrika, Fernando, & Ranaweera, 2009) while carotenoid content of papaya (13.8 mg/100 g dry pulp) was low compared to mango, carrot and tomato (Pamplona, 2003). Thermal treatment induced the degradation of carotenol fatty acid esters of xanthophylls while freezing and canning of papaya slices led to significant decreases in the total carotenoids quantified by HPLC (Cano, de Ancos, & Lobo, 1996). Irrespective of the variety, the guava blended papaya fruit leather had very meagre amount of 0.001mg /100mg of ß-carotene. This indicated that use of open pan boiling destroyed the minimal amount that existed in the papaya guava blend. Therefore this method is not suitable if ß-carotene is to be retained through processing.

### 3.2. Sensory analysis

The sensory evaluation was conducted on the two products of papaya leather prepared from the two types of papayas which were acting as basic ingredients and guava in different proportions. Each panelist was given the time to assess the organoleptic attributes of the two different leather product and the five point hedonic scale evaluation form to fill according to their preference. Conferring to the results obtained, the Hawaiian papaya-guava leather (60% of Hawaiian papaya pulp and 40% of guava pulp) was the most preferred by panelists. The appearance and flavor of the papaya leather from Hawaiian variety were more preferred than those of the Mexican variety; however in terms of color Hawaiian papaya-guava leather were the most liked. But statistical analysis indicated that difference between the two papaya varieties in terms of the appearance, flavor, taste, texture, color and overall acceptability of resulting papaya-guava leather was not significant neither at 5% nor at 1% level of significance. Fig 4 shows the descriptive sensory attributes of the two samples studied.

## 4. Conclusions

Fruit leathers are made from fruit puree and other ingredients such as sugar and citric acid to enhance the acceptability and storage stability. They are nutrient dense; their calories and acceptability can be further enhanced by the addition of nuts or spices. Blending of fruit purees to make fruit leathers enhances the nutritional quality apart from preventing postharvest losses. Vitamin C loss in the processed fruit leather ranged from 33.5% to 45.6% of the original level. Thus nearly half of the vitamin C was lost upon processing of leather which was quiet high. The study proved that there was no statistical difference (p-value>0.05; p-value>0.01) in sensory attributes between Hawaiian papaya leather and Mexican papaya leather. Hence, underutilized fruits such as papaya and guavas can be successfully blended and processed into organoleptically acceptable fruit leather as revealed through this study. However further studies would be necessary to appraise available technologies to improve the vitamin C stability in papaya-guava leather.

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# INVESTIGATION OF CHANGES IN ANTIOXIDANT ACTIVITIES OF CARAMELIZATION PRODUCTS UNDER VARIOUS TIME REGIMES AND pH RANGES

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**ABSTRACT**

Caramelization is a process of heating sugars to produce brown color and typical caramel flavor which is most widely used in food industry as a natural food color, flavor and antioxidant agent. These properties of caramelization products (CPs) are heavily dependent on type of sugar, time of heating and pH range. A study was conducted to prepare CPs utilizing different type of sugars (dextrose; fructose; Liquid glucose; sucrose); and to investigate the changes in products characteristics at different time and pH reaction conditions using response surface methodology. The ranges of processing variables selected for this study were: time, 30-150 min and pH, 4-10. The experimental values of reducing sugars, browning intensity, reducing power and antioxidant activity showed that response variable was mainly dependent on increase in time of processing regardless of sugar type. Browning reactions occurred to a greater extent at alkaline pH while dextrose was more reactive to caramelization than other sugars at neutral pH. After 150 min, dextrose, fructose, L-glucose and sucrose were degraded to 46.9%, 34.9%, 23.4% and 39.7%, respectively. CPs from hexose sugars rendered the greater reducing power, compared with CPs from pentose. DPPH radical scavenging activity was observed in descending order such as: fructose>dextrose>sucrose>L-glucose ($p \leq 0.01$). The results of this study demonstrated that dextrose and fructose are a good source of natural antioxidant involving caramelization and can be potentially used as new food ingredients to enhance the shelf life of food products.

## 1.Introduction

Caramelization is the thermal degradation of sugars leading to the formation of volatiles and brown colored products. It is a type of non-enzymatic browning and may be carried out in the presence of acid, alkali and salt or without these at a temperature more than 80 oC and pH range of 3-12 (Davies and Labuza, 2005). Caramels are formed by heating low molecular weight carbohydrates, such as dextrose or starch hydrolysate under a variety

of reaction conditions. Caramels; dark brown to black viscous liquids with the characteristic odor of burnt sugar and a somewhat bitter taste; are among the oldest colorants known to be added to human food. Their use accounts for about 95% by weight of the permitted color additives used in food. Never the less, because of their complex composition, caramels color have remained chemically rather ill defined (Houben and Penninks, 1994).

Caramel colors have been in general use of more than 100 years, but only recently extensive effort has been made for characterize these color additives to specify better the materials being marketed today (Fadel and Farouk, 2002). Caramels are found in almost every kind of industrially produced food, including: beer, brown bread, buns, chocolate, cookies, brandy, chocolate flavored flour-based confectionery, coatings, decorations, fillings and toppings, chips, dessert mixes, doughnuts, fish and shellfish spreads, frozen desserts, glucose tablets, cough drops, gravy browning, ice cream, jams, milk desserts, pancakes, pickles, sauces and dressings, soft drinks (especially colas), stouts, sweets. This importance of caramels is due to their stabilizing, emulsifying, free radical scavenging, antimicrobial and antioxidative properties (Faraji and Lindsay, 2005; Tsai, 2009). Addition of fructose, glucose, maltose or citrate to the raw material increases contributions to volatile formation during baking and heating but Millard and caramelization reactions are also responsible for flavor formation in baked cereal products (Rehman *et al*., 2006).

The caramelization of carbohydrate polymers and their mixtures with low molecular weight sugars is of interest for food processing not only because of the caramel flavor and color, but also because the changes in sugar structure and the liberation of water during the caramelization reaction (Kroh, 1994). The exact reaction conditions and chemical reactants used are selected to give the caramel its desired characteristics. The influence of reaction conditions on the quality of caramels is continuously considered to be the problem of present interest. Composition of caramel from the qualitative point of view is independent of the sugar used but it is influenced by the method employed for its preparation. Several reports documented the effect on caramelization and its results of such parameters like temperature, mode of its application, time, pH, pressure, atmosphere and catalyst added (Sikora *et al*., 1989; Ajandouz and Puigserver, 1999).

The response surface methodology is a mathematical and statistical approach which has been widely used to evaluate the response of multivariate parameters during modeling of variable processing conditions for food production. The objective of this study was to investigate the influence of processing time and pH conditions for production of caramel products and their color and antioxidant characteristics using response surface approach

## 2. Materials and methods

### 2.1. Chemicals

Fructose, Potassium Ferricsyanide, Ferric Chloride, Di-Sodium Phosphate, Mono Sodium Phosphate and Trichloroacetic were purchased from Merck. 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) was purchased from sigma. Dextrose and Liquid glucose were kindly supplied by Rafan maize products; while sucrose was supplied by Crescent Sugar Mills, Faisalabad, Pakistan.

### 2.2. Preparation of Caramelized Products (CPs)

Solutions of sugars were prepared by mixing with 0.05 M phosphate buffer of a pH range of 4-10. 10 mL of each sugar solution was transferred to a screw-caped test tube and was subjected to heating for time duration range of 30-150 minutes. At the heating time

designated the samples was taken out and cooled in ice water immediately and was stored at 4 $^{o}$C for further analysis.

### 2.3. Browning Intensity Determination

Browning intensity of the CPs was measured by the spectrophotometer at $A_{420}$ (Benjakul *et al.* 2005). Appropriate dilution was made for all the samples using distilled water. The absorbance showed the browning intensity of the caramelization products.

### 2.4. Determination of Reducing Sugars Concentration

Reducing sugars in CPs were measured according to the method of Benjakul et al. (2005) Fifty-fold dilution was made for all samples before analysis. Standard curves were prepared using the individual sugars. The changes in reducing sugar were expressed as the relative concentration (%) in comparison with the original content.

### 2.5. Determination of reducing power

The reducing power of Cps was measured as described by Benjakul et al. 2005 with slight modification. 0.5 mL of each sample was mixed with 0.5 mL of 0.2 M sodium phosphate buffer, pH 6.6 and 0.5 mL potassium ferric cyanide. The reaction mixture was incubated at 50 $^{o}$C for 20 minutes and 0.5 mL of 10% (w/v) trichloroacetic acid (TCA) was added. Thereafter, 2 mL of distilled water and 400 µL of 0.1% (w/v) ferric chloride were added to mixture and then absorbance was measured.

### 2.6. DPPH Radical Scavenging Activity

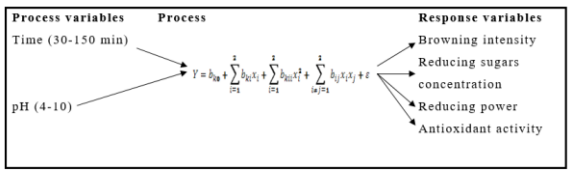
The radical scavenging activity was measured according to the method of Tsai et al. 2008. One millimeter of the freshly prepared 1 mM DPPH solution was added to the samples. The solution was than mixed vigorously and allowed to stand at 25 $^{o}$C for 30 minutes. The absorbance of the mixtures was read at 517 nm using a UV-1601 spectrophotometer. The control was prepared in the same way, except that distilled water was used instead of CPs samples. For the blank, the assay was conducted in the same manner but distilled water was added instead of DPPH solution. The percentage of DPPH radical scavenging activity was calculated by following the method of Singh and Rajini (2004) as follows:

$$\text{Radical scavenging activity \%} = (1-(A_{\text{sample (517 nm)}} / A_{\text{control (517 nm)}})) \times 100 \quad (1)$$

### 2.7. Experimental Design and Statistical Analysis

Response surface modeling for quality changes involves multiple process control input parameters and selected product output properties. Response surface methodology was applied to determine the best combination of process variables for the production of caramelized products. This experimental study was carried out to determine the effects of independent processing variables on CPs using the faced central composite design (CCD). The effect of time (30–150 min) and pH (4–10) on the response values of CPs was examined. For better accuracy and simplification of result interpretation, the coded multiple regression coefficients were used and reconverted into original values at the end of experiment using MATLAB® (Ver. 7.9.0) software (Mathworks, Inc., Natick, USA). The coded coefficients at three levels used in this study were –1 (lowest level), 0 (medium level) and 1 (highest level), respectively (Table 1).

The following empirical ‘‘black box’’ modeling presents the relationships among process and response variables:



The expression inside the ''black box'' represents browning intensity, reducing sugars concentration; reducing power and antioxidant activity when the value of *i* is changed from 1 to 4; $b_{ko}$; $b_{ki}$; $x_{kii}$ ; and $b_{kij}$ represent the constant and coefficients of linear, quadratic and interactive effects, respectively; $X_i$ ; $X^2_i$ and $X_iX_j$ represent the linear, quadratic and interactive effects of the independent variables, respectively, and ε is the random error primarily to account for the inability to determine the true model (Reyes-Moreno *et al*., 2003).

## 3.Results and discussions

### 3.1. Browning Intensity of CPs

The final stage of the browning reaction in CPs at pH (4, 7 and 10) and time (30, 90 and 150 min) was monitored by the increase in absorbance at 420 nm (Figure 1). The analysis of variance (ANOVA) for browning intensity of caramelized products from different sugars at time and pH reaction conditions has been presented in Table 2. Increase in browning was generally observed as the heating time increased. Regardless of sugar type, browning reactions occurred to a greater extent at alkaline pH (10), compared with at neutral pH (7). Browning at pH 7 increased continuously with increasing heating time; whereas browning occurred sharply within the first 30 min at pH 10. Subsequently, the browning was increased at a slower rate. The result is in agreement with Phongkanpai1 et al. (2006) which observed browning development of fructose at alkaline pH ranges. At pH 7, dextrose was more reactive to caramelization than the other sugars as indicated by the development of browning (Figure 1A). However, fructose was more likely to undergo browning via caramelization at pH 10 (Figure 1B). The differences in browning found among all sugars tested might be related to their different relative structural stability, including mutarotation, opening of the hemiacetal ring and enolization of the sugar. Browning development is influenced by the type of sugar and pH and the rate of color development decreased as the pH decreased. Buera et al. (1987) reported that rates of browning development of reducing sugars via caramelization processes were in the descending order: fructose > xylose > lactose > maltose > glucose. Thermolysis causes dehydration of sugar molecules with the introduction of double bonds or formation of anhydro rings. Introduction of double bonds leads to unsaturated rings and conjugated double bonds absorb light and produce color. Unsaturated rings will condense to polymers leading to the development of color (Benjakul *et al*., 2005).

### 3.2. The Loss of Sugars

The increased degradation of all sugars was observed as the heating time increased (Figure 2). The rate of sugar degradation was much greater under alkaline pH conditions, compared with a neutral pH. At pH 7, a slight decrease in sugar was found during the first 30 min of heating. Thereafter, sugars, especially fructose and sucrose, underwent more extensive degradation as evidenced by the marked decrease in reducing sugar content (Figure 2B; 2D). The analysis of variance (ANOVA) for reducing sugars concentration in caramelized products from different sugars at time and pH reaction conditions has been presented in Table 3. Fructose and sucrose decreased to 73.5% and 75.4% after heating time for 150 min. At pH 10, sharp degradation was observed in all sugars during the first 30 min of heating. Subsequently, sugars underwent degradation gradually up to 150 min. Among all of the sugars tested, dextrose was degraded to a smaller extent, compared to the other (Figure 2A). Higher levels of degradation of both fructose and dextrose occurred at 100 ºC under alkaline conditions ((Benjakul *et al*., 2005; Ajandouz and Puigserver, 1999; Ajandouz *et al*., 2001). After 150 min,

glucose, fructose, L-glucose and sucrose had degraded to 46.9%, 34.9%, 23.4% and 39.7%, respectively. From these results, it was determined that the rate of degradation was dependent upon pH and the type of sugar involved.

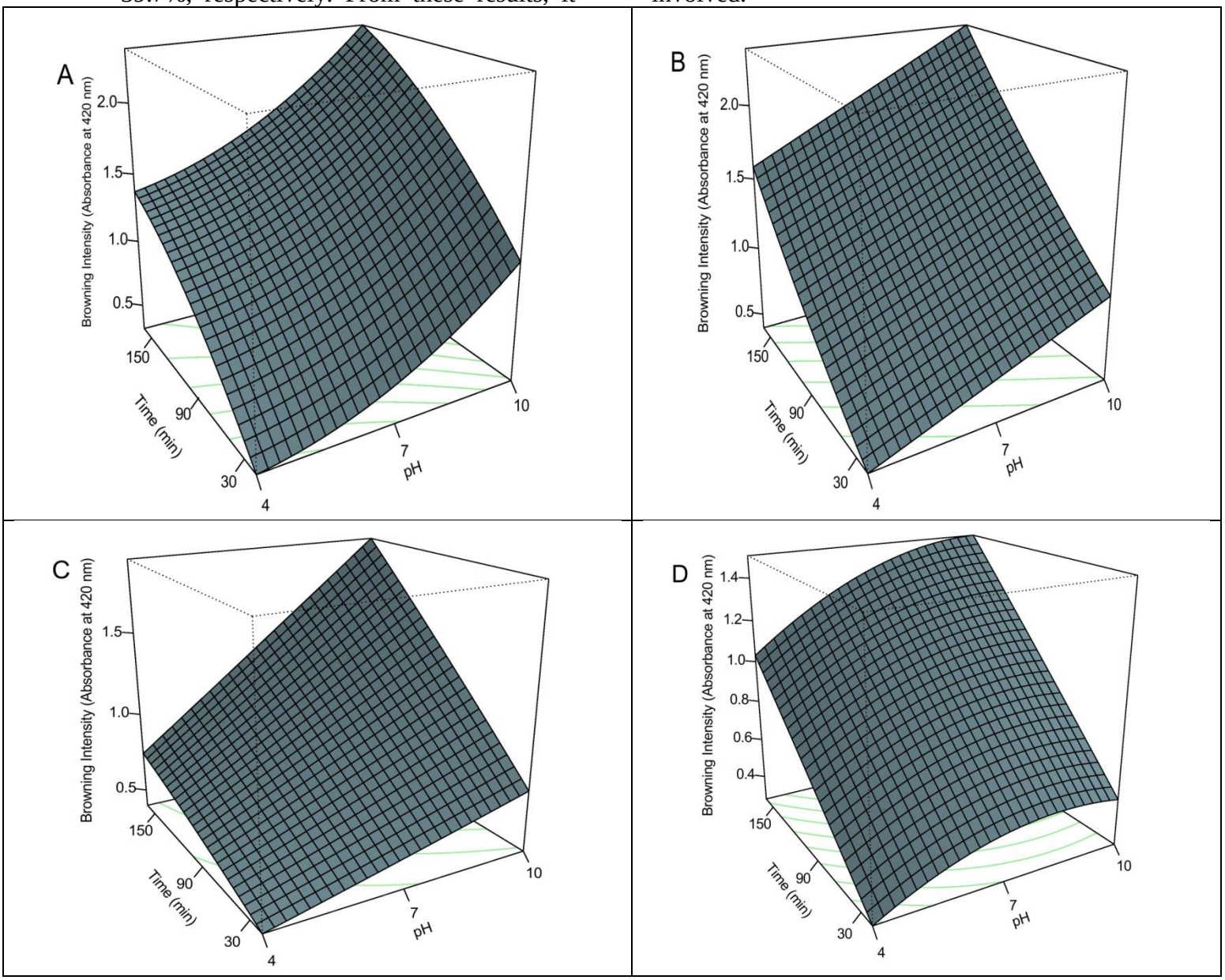



**Figure 1**. Mutual Interaction effect of time and pH reaction conditions on browning intensity of caramelized products for sugar type (A) Dextrose (B) Fructose (C) L- Glucose (D) Sucrose

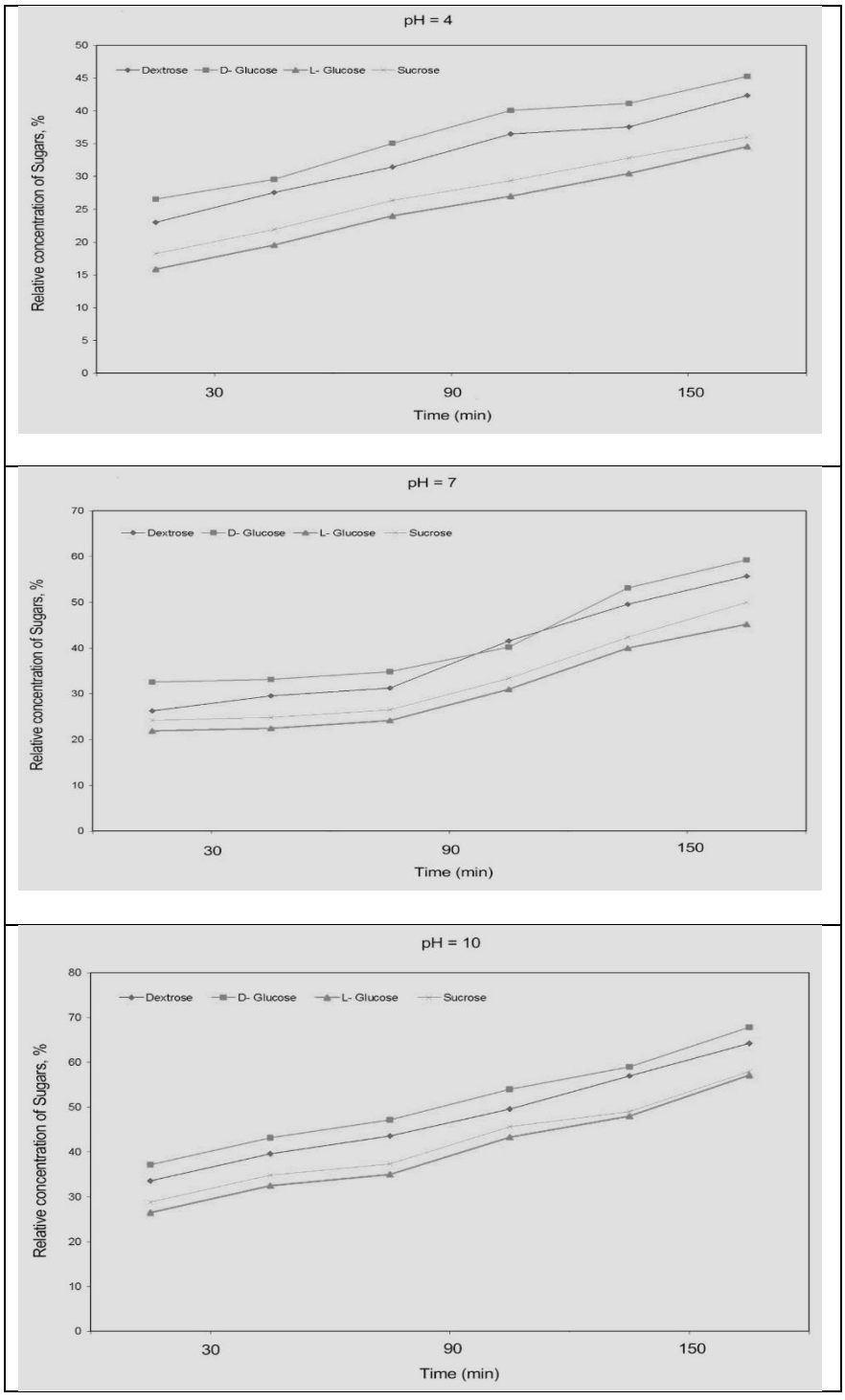



**Figure 2.** Effects of time and pH reaction conditions on relative concentration for sugar types in caramelized products

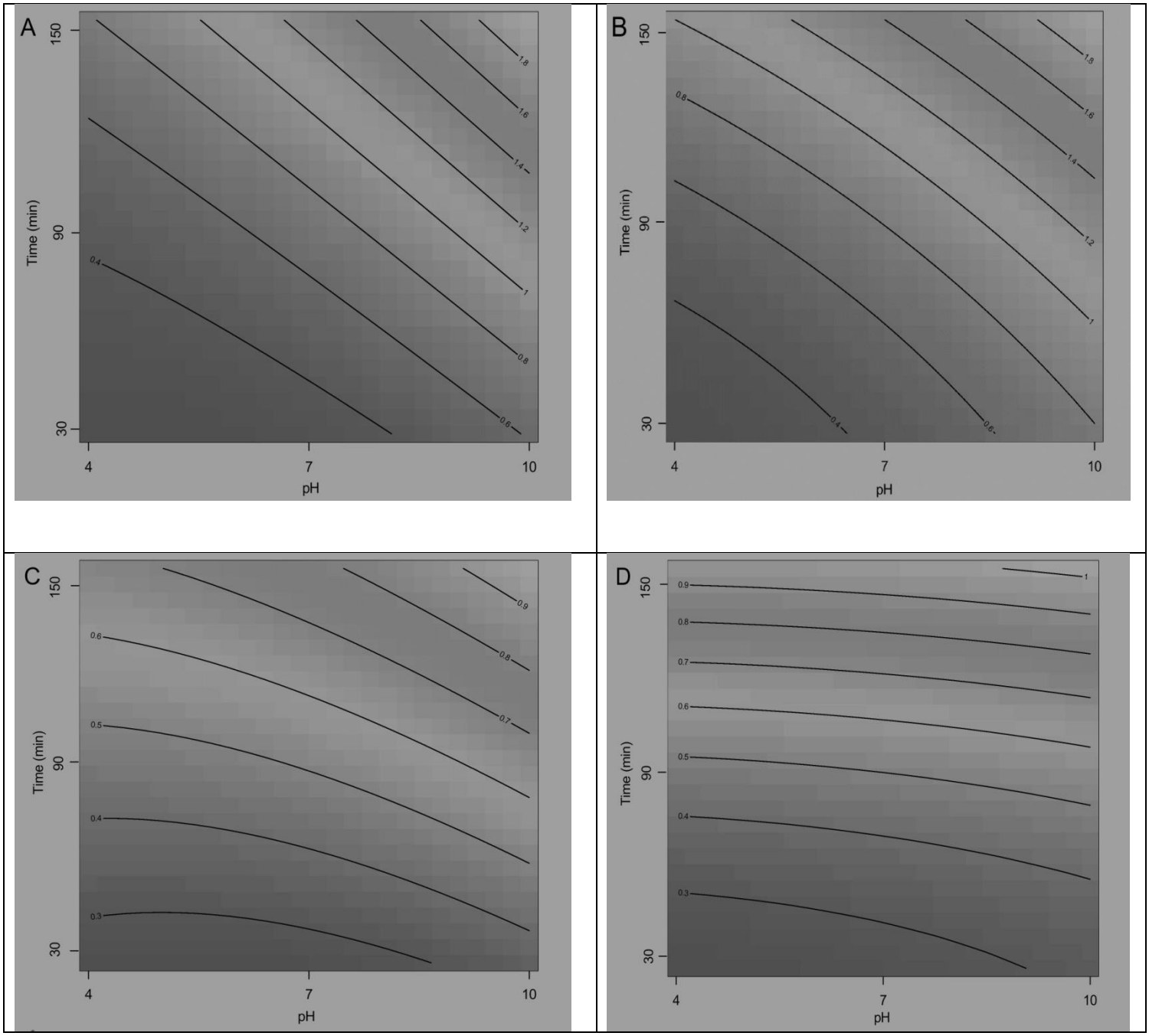



**Figure 3.** Mutual Interaction effect of time and pH reaction conditions on reducing power of caramelized products for sugar type (A) Dextrose (B) Fructose (C) L- Glucose (D) Sucrose

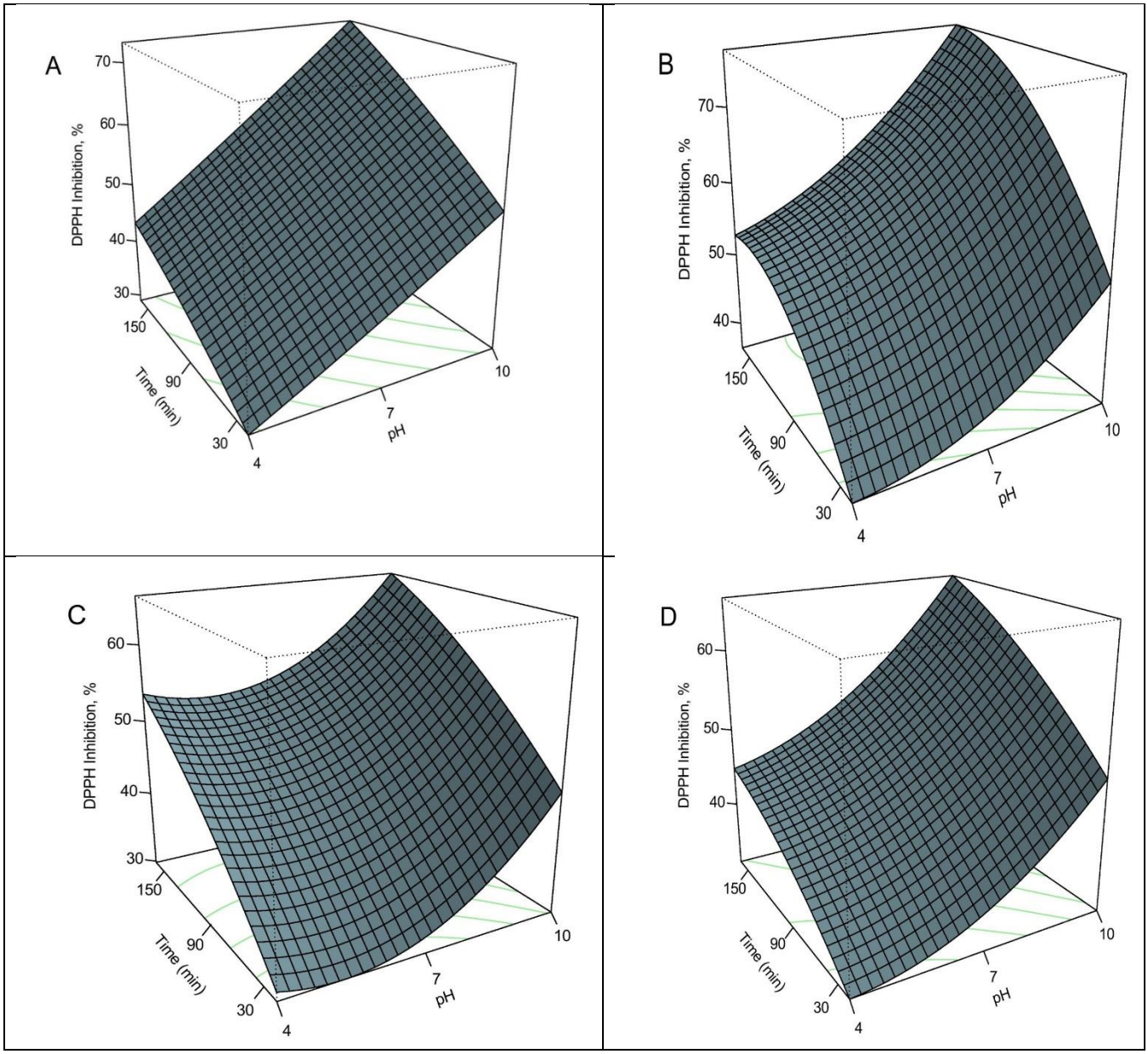



**Figure 4.** Mutual Interaction effect of time and pH reaction conditions on antioxidant activity of caramelized products for sugar type (A) Dextrose (B) Fructose (C) L- Glucose (D) Sucrose

### 3.3. Reducing Power of CPs

Reducing power of CPs from different sugars prepared by heating at pH 3, 7 and 10 for different times is depicted in Figure 3. Under neutral conditions, the reducing power of CPs, as indicated by the increase in absorbance at 700 nm, increased linearly as the heating time increased (Figure 3). Fructose CPs showed the highest reducing power, compared with CPs from other sugars (Figure 3B). The analysis of variance (ANOVA) for reducing power of caramelized products from different sugars at time and pH reaction conditions has been presented in Table 4. For CPs prepared under alkaline conditions, reducing power

increased exponentially with increasing heating time. A sharp increase in reducing power was observed when heating was conducted for up to 30 min. Heating for a longer time did not result in increased reducing power. Generally, CPs from fructose exhibited the highest reducing power (Figure 3B) and CPs from dextrose (Figure 3A) showed higher reducing power than those from sucrose and L-glucose (Figure 3C; 3D). Thus, it can be concluded that CPs from hexose sugars rendered the greater reducing power, compared with CPs from pentose. During heating of sugar solutions, especially under alkaline conditions, reducing compounds might be formed and these could exhibit antioxidative activity. Antioxidative activity of Maillard reaction products was associated with reducing power (Yen and Hsieh, 1995). The reducing power of CPs might be due to hydrogen-donating ability (Shimada *et al*., 1992; Benjakul *et al*., 2005;).

### 3.4. DPPH radical scavenging activity

DPPH radical scavenging activity of CPs from sugars prepared by heating under neutral and alkaline condition is shown in Figure 4. The analysis of variance (ANOVA) for antioxidant activity of caramelized products from different sugars at time and pH reaction conditions has been presented in Table 5. DPPH radical scavenging activity of CPs prepared under neutral conditions increased linearly as the heating time increased. Among CPs from all sugars tested, those from sucrose showed the highest activity (Figure 4D). CPs from dextrose were found to exhibit to lowest activity, compared to CPs from other sugars (Figure 4A). For CPs prepared under alkaline conditions, an exponential increase in DPPH radical scavenging activity was observed with increasing heating time. CPs from fructose exerted greater DPPH radical scavenging activity compared to CPs from other sugars (Figure 4B). DPPH radical scavenging activity was in the descending order: fructose>dextrose>sucrose> L-glucose ($p \leq 0.01$). From the result, it was noted that DPPH radical scavenging activity of CPs prepared under alkaline conditions was approximately five-fold greater than that of CPs prepared under neutral conditions. The higher radical scavenging activity of CPs prepared at pH 10 was coincidental with the higher reducing power, browning and intermediate formation. DPPH is one of compounds that possess a proton free radical with a characteristic absorption, which decreases significantly on the exposure to proton radical scavengers (Yamaguchi *et al*., 1998). It was found that CPs was able to reduce the DPPH radical to the yellow-coloured diphenylpicrylhydrazine. The reduction of alcoholic DPPH solution in the presence of a hydrogen- donating antioxidant is due to the formation of the non-radical form, DPPH-H (Shon *et al*., 2003). Thus it was suggested that either intermediates or the final brown polymer could function as hydrogen donors. Kirigaya and fellows (1968) found that antioxidant activity increased with increasing color intensity. However, Rhee and Kim (1975) reported that effective antioxidant compounds were formed at an earlier stage of browning reactions. Therefore, CPs, especially those from caramelization under alkaline conditions, exhibited antioxidant activity.

**Table 1.** Coded and Actual Levels of Independent Variables Used for Production of Caramelized Products (CPs) as Determined by The Central Composite Design (CCD).

| Independent variable | Coded levels | | |
|---|---|---|---|
| | -1 | 0 | 1 |
| **Time, min** | 30 | 90 | 150 |
| **pH** | 4 | 7 | 10 |

**Table 2**. Mean Sum of Squares for Browning Intensity of Caramelized Products from Different Sugars at Time and pH Reaction Conditions.

| Source of variation | df | Dextrose | Fructose | L- Glucose | Sucrose |
|---|---|---|---|---|---|
| **Intercept** | 5 | 0.73** | 0.63** | 0.39** | 0.32** |
| **pH(A)** | 1 | 1.27** | 0.7** | 0.9** | 0.18* |
| **Time(B)** | 1 | 2.34** | 2.44** | 0.86** | 1.33** |
| **A×B** | 1 | $5.476^{NS}$ | $0.011^{NS}$ | 0.16** | $0.015^{NS}$ |
| **$A^2$** | 1 | $8.103^{NS}$ | $4.253^{NS}$ | $5.344^{NS}$ | $0.06^{NS}$ |
| **$B^2$** | 1 | $6.572^{NS}$ | $5.265^{NS}$ | $1.107^{NS}$ | $3.523^{NS}$ |
| **Residual** | 5 | 0.018 | 2.058 | 7.689 | 0.011 |

**Significant at 0.001 level
*Significant at 0.01 level
$^{NS}$ Non-significant

**Table 3.** Mean Sum of Squares for Relative Sugar Concentration in Caramelized Products from Different Sugars at time and pH Reaction Conditions

| Source of variation | df | Dextrose | Fructose | L- Glucose | Sucrose |
|---|---|---|---|---|---|
| **Intercept** | 5 | 210.53** | $274.50^{NS}$ | 157.99** | 148.16** |
| **pH(A)** | 1 | 315.81** | $276.62^{NS}$ | 253.5** | 240.67** |
| **Time(B)** | 1 | 676.28** | $529.78^{NS}$ | 479.54** | 459.2** |
| **A×B** | 1 | $13.9^{NS}$ | $13.18^{NS}$ | $16^{NS}$ | $6.25^{NS}$ |
| **$A^2$** | 1 | $17.19^{NS}$ | $181.52^{NS}$ | $7.44^{NS}$ | $5.28^{NS}$ |
| **$B^2$** | 1 | $21.70^{NS}$ | $280.8^{NS}$ | $27.54^{NS}$ | $24.64^{NS}$ |
| **Residual** | 5 | 8.60 | 247.9 | 6.79 | 4.49 |

**Significant at 0.001 level
$^{NS}$ Non-significant

**Table 4.** Mean Sum of Squares for Reducing Power of Caramelized Products from Different Sugars at Time and pH Reaction Conditions.

| Source of variation | df | Dextrose | Fructose | L- Glucose | Sucrose |
|---|---|---|---|---|---|
| **Intercept** | 5 | 0.51** | 0.42** | 0.096** | 0.16** |
| **pH(A)** | 1 | 0.91** | 0.82** | 0.056** | $0.01^{NS}$ |
| **Time(B)** | 1 | 1.41** | 1.15** | 0.41** | 0.75** |
| **A×B** | 1 | 0.18** | 0.054* | 9.312* | $1.6^{NS}$ |
| **$A^2$** | 1 | 0.029** | 0.059* | $4.253^{NS}$ | $4.947^{NS}$ |
| **$B^2$** | 1 | 0.025** | $0.012^{NS}$ | $3.942^{NS}$ | 0.031* |
| **Residual** | 5 | 1.231 | 6.167 | 8.642 | 4.387 |

**Significant at 0.001 level

*Significant at 0.01 level

NS Non-significant

**Table 5.** Mean Sum of Squares for Antioxidant Activity of Caramelized Products from Different Sugars at Time and pH Reaction Conditions.

| Source of variation | df | Dextrose | Fructose | L- Glucose | Sucrose |
|---|---|---|---|---|---|
| **Intercept** | 5 | 315.19** | $354.71^{NS}$ | 212.88** | 197.37** |
| **pH(A)** | 1 | 1027.83** | 622.2* | 276.08** | 543.4** |
| **Time(B)** | 1 | 533.36** | 1124.77* | 691.23** | 421.68** |
| **A×B** | 1 | $13.43^{NS}$ | $16.81^{NS}$ | $1.21^{NS}$ | $6.0^{NS}$ |
| **$A^2$** | 1 | $0.5^{NS}$ | $6.47^{NS}$ | 92.36** | $15.09^{NS}$ |
| **$B^2$** | 1 | $0.61^{NS}$ | $1.85^{NS}$ | $11.9^{NS}$ | $2.14^{NS}$ |
| **Residual** | 5 | 2.21 | 69.98 | 4.34 | 4.44 |

**Significant at 0.001 level

*Significant at 0.01 level

NS Non-significant

## 4. Conclusions

The results of this study demonstrated that the caramelized products can be produced with good quality to be used as color, flavor and antioxidant additive. Among the experimental conditions used in this study, time (~150 min) and alkaline pH was found most significant for browning intensity, reducing power and antioxidant activity. Based on the results of this study, it can be concluded that caramelized products can be successfully produced having high tinctorial strength and antioxidant activity by utilizing local resources. Our results suggest that dextrose and D- glucose are a good source of natural antioxidant involving carameliation and can be potentially used as new food ingredients to enhance shelf life of food.

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# OZONE FOOD STORAGE SUPPLIED BY PHOTOVOLTAIC ENERGY

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**ABSTRACT**

In Algeria and in several Third World countries, there are many isolated sites that are not yet connected to the conventional electrical grid. They face mostly problems such as food storage, which is a real constraint for rural residents. The purpose of this paper is to design and implement at a lower cost a system comprising a photovoltaic system supplying an ozone generator for the disinfection of a food storage room in an isolated site to increase the shelf life of the food products. An experimental study was conducted using several fruits and vegetables that were placed in an ozone treated room. A comparative study using a similar control untreated room has shown that such a system allows long-term storage with low energy consumption of nearly 16 W/m$^2$.

## 1.Introduction

In recent decades, various cultural, industrial and economic activities have led to increased electricity consumption, raising concerns about greenhouse gas emissions and the reliability of electricity supply. The photovoltaic (PV) system advantages include a long service life, reduced maintenance, ease of installation and no fuel consumption; the major disadvantage is the low production by cloudy weather

The geographical location of Algeria gives it a high solar potential and good exposure to solar energy. By receiving an average of 3000 hours/year of solar radiation, Algeria has the highest solar potential in the Mediterranean basin (169440 TWh / year) (Bey et al., 2016).

In addition, despite the government efforts to increase the electrification rate, some households in rural areas are deprived of electricity. The high cost of the investments needed to expand public networks as well as the limited needs of the remote areas concerned will continue to hinder their connection in the medium term. This is why photovoltaic systems in isolated sites are an interesting alternative (Korsaga et al., 2018).

On the other hand, these households are confronted with the problem of food storage of fruits and vegetables. Generally food storage is carried out in cold rooms at low temperature (below 10 °C) whose electrical operating power is relatively high (2000 W for a volume of 20 m$^3$), requiring thus a great amount of electrical energy. Therefore, because of the high cost for implementing the necessary equipment for cold rooms, such as air conditioning, accessories, etc.., the high electricity consumption and the precarious financial situation of third world countries, it is difficult to build cold rooms with its requested electrical energy in isolated sites.

This is why ozone treatment of non-air conditioned food storage rooms could be an

effective and economical solution for the conservation of agri-food products.

One of the important usages of ozone in agriculture is the post-harvest treatment of harvested crops. Ozone can be applied to foods as a gas or as a dissolved form in water. The main purposes of ozone application at the postharvest stage are inactivation of bacterial growth (Sharma, et al., 2002; Achen & Yousef., 2001; Kim & Yousef.,2000; Kim & Yousef., 1999; Roya et al., 2016), prevention of fungal decay (Palou et al., 2002; Perez et al., 2002), destruction of pesticides and chemical residues (Hwang, et al., 2001; Ong et al., 1999), and control of storage pests (Mendez et al., 2003; Kells et al., 2001).

Ozone is increasingly used for the storage of fruits, vegetables, flowers, meat, fish, cheese, etc ... for strengthening the quality of conservation of the market, control of pathogens, molds, fungi, yeasts and other micro -organisms. In (Ewell et al., 1938; Pérez et al., 2002) it is stated that the shelf life of strawberries can be doubled if a dosage of 2-3 ppm (particles per million) of ozone is applied regularly for a few hours a day. Fruits and vegetables such as apples, potatoes, tomatoes and many more can be disinfected and kept longer thanks to ozone. In (Skog & Chu., 2001) it is indicated that the application of ozone at reduced concentrations in cold rooms on cucumbers, mushrooms, apples and pears for different temperatures can increase storage time.

Moreover, in (Brahami et al., 2015) a photovoltaic solar system is described for supplying an ozone generator, which has been used for water treatment. It has been shown that the optimization of the orientation angles of photovoltaic panels can increase the efficiency of the ozone water treatment system despite the climatic disturbances due mainly to the passage of clouds.

The aim of this work is twofold: to increase the storage time of food in rooms located in isolated sites that do not have air conditioning system using ozone disinfection, while ensuring an autonomous power supply by photovoltaic energy. Different fruits and vegetables were tested in a closed room treated with ozone.

## 2. Materials and methods

Figure 1 shows the overall experimental system used for analyzing food storage in an ozone treated atmosphere supplied by a PV power system. The experimental setup consists of two separate parts: the solar energy system and the ozonation system.

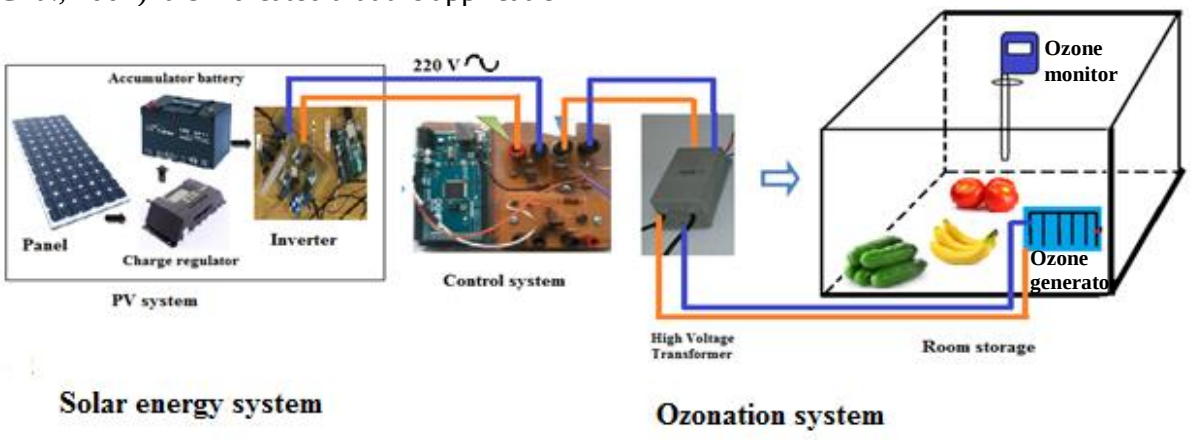
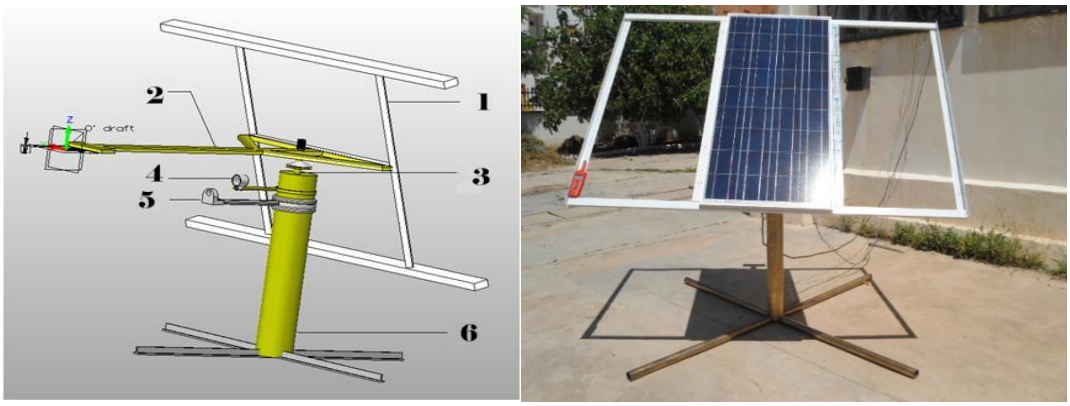



**Figure 1.** Schematic representation of the experimental setup

### 2.1. Solar energy System

As shown in (Fig.1), the solar energy system includes a photovoltaic generator producing a power of 135 W, a charge controller (12/24 V, 20 A) to keep the voltage constant at 12V, an 80 Ah storage battery used to store the energy and a single-phase inverter. In this work, the solar PV system was designed to allow the solar panel to undergo a horizontal and a vertical rotation movement to follow the sun and thus optimize the surface exposed to sunlight (Fig.2). Therefore, an optimal panel position can be determined corresponding to a high level of power generation. Preliminary experiments made it possible to define the ideal position of the panel which should be oriented towards the south (corresponding to zero east-west angle), with an incline south to north angle varying from $\beta = 40°$ to $\beta = 50°$ in Sidi Bel Abbes city, Algeria (Brahami et al., 2015).




**(a )** **(b)**

**Figure 2**. Photovoltaic system
**a)** General view of the mechanical structure of the PV panel
b) Photography of the PV panel
1. Framework supporting the PV panel, 2: Limit switch of south-north Axis; 3: East-west orientation Pulley; 4: Motor for East-west orientation; 5: Jack Support for north-south orientation; 6: Cylinder supporting the structure.

### 2.2. Ozonation process

The ozone treatment was carried out inside a metal enclosure of dimensions 2x1.2x1 $m^3$ inside which was placed an ozone generator fixed on the upper wall. An identical metal enclosure without ozone generator was used as a control room. A portable ozone monitor (O3 Technologies) was used to measure the ozone concentration in the treated room, in ppm.

The best method for generating ozone is to pass oxygen ($O_2$) through a plasma produced by Dielectric Barrier Discharge (DBD). A planar surface DBD reactor was developed, comprising a dielectric barrier made of Bakelite with dimensions 190 x 140 x2 $mm^3$ (Fig.3). The electrodes are made of aluminum adhesive strip placed on the opposite sides of the plate. The high voltage electrode consists of 10 strips of dimensions 170 x 3 $mm^2$ while the ground electrode is an aluminum strip bonded to the other face of dimensions 170 × 120 $mm^2$.

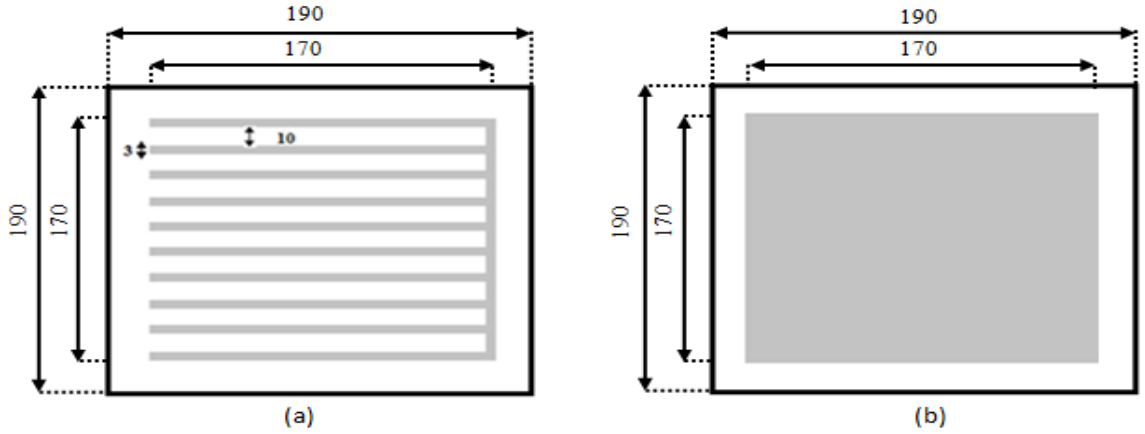



**Figure 3.** Bottom (a) and top (b) view of the flat ozone generator (all dimensions in mm)

### 2.3. Experimental procedure

Fresh foods bought at the local market were placed in both rooms and kept in storage for a period of 20 days. Photos were taken at regular intervals for visual analysis of food quality. In addition, mass weighing were taken to estimate food weight loss over time.

The weight loss was calculated using the following formula:

$$\Delta m = (m_i - m_f)/m_i \quad (1)$$

with

$m_i$ : initial mass (1st Day)

$m_f$: final mass (20th Day)

The typical ozone concentrations typically used for the treatment in cold food storage rooms should be in the range 2-7 ppm (Ewel et al, 1938). Consequently, a time control system has been developed using Arduino card programming to control the On /Off time periods of the ozone generator to ensure a continuous ozone concentration ranging between 2 and 7 ppm.

To achieve these concentrations, preliminary tests were carried out to determine the optimal operation periods of the ozone generator. All the experiments were performed under stable conditions of temperature (15 ± 5 ° C) and humidity (50 ± 10%) inside the storage room

### 3.Results and discussions

The results of the preliminary experiments are plotted in (Figs.4 and 5), represent the evolution of the ozone concentration during the operation of the ozone generator (Period On) and the decline of the concentration during the shutdown (period Off) of the generator, respectively.

As shown in Figure 4, the time duration required to reach an ozone concentration of 7 ppm is 10 seconds. Moreover, the results presented in (Fig.5) show that the duration during which the concentration decreases from 7 to 2 ppm according to the humidity and the temperature conditions is 15 minutes.

Based on these results, the time control system of the ozone generator has been set to maintain an ozone concentration between 2 and 7 ppm, corresponding to an operating time of 10 seconds applied at shutdown intervals of 15 minutes.

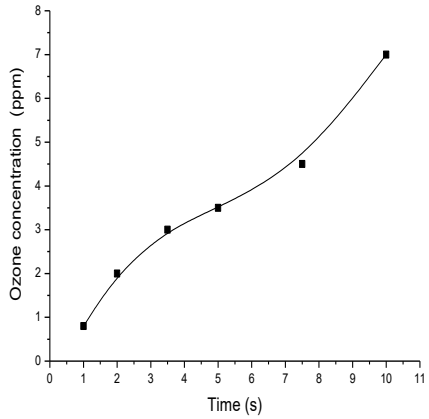



**Figure 4.** Evolution of the ozone concentration as a function of the operating time of the ozone generator

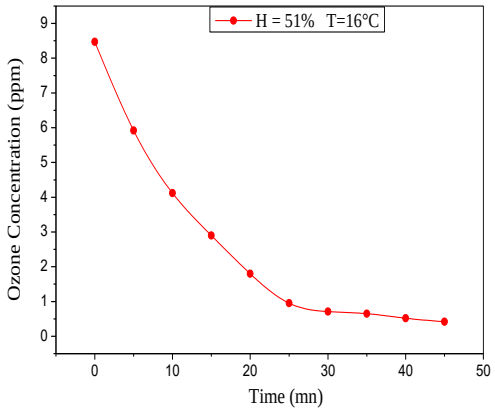
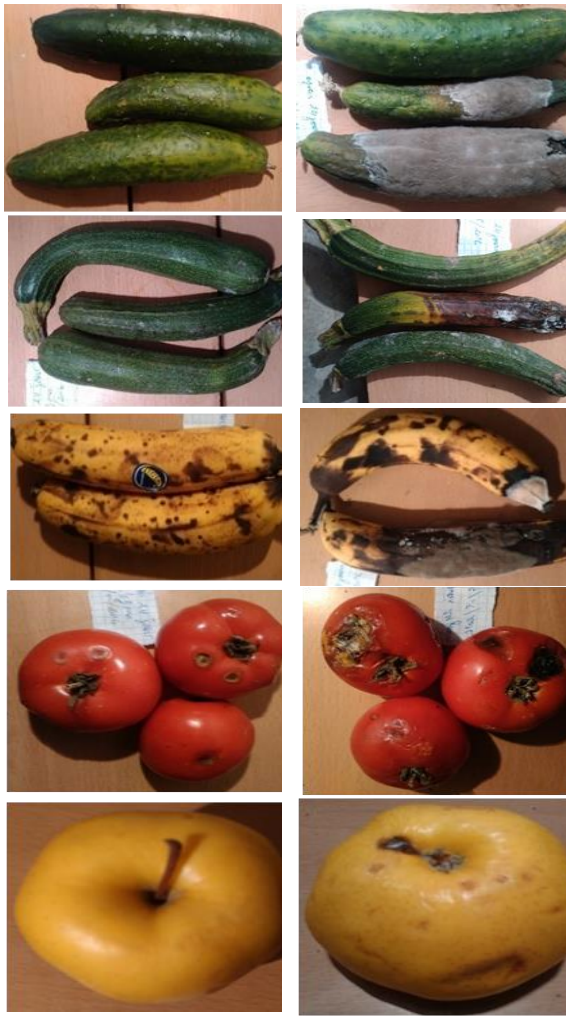



**Figure 5.** Decline of the ozone concentration as a function of the time during the shutdown of the ozone generator

Five food products were tested: tomato, cucumber, zucchini, apple and banana. (Fig. 6) shows photographs taken after 24 days of storage, in both treated and untreated enclosures.

These results clearly show that the visual aspect of the products stored in the ozone-treated room is much better. Indeed, the ozone is a powerful oxidizer that has been recommended by several researchers (Liew et al, 1994; Sarig et al , 1996) to reduce the decomposition of the product and prolong the storage period, eliminating bacteria and stopping their development. Ozone also reacts with ethylene, the gas responsible for the ripening of fruits and vegetables because it causes damage and increases decomposition ( Skog, L. J & Chu, 2000 ; Suslow, 2004; Tayyari et al, 2017). In addition, it is mentioned in several studies (Smilanick et al, 2003; Tuffi et al , 2012; Montesano et al , 2004) that the exposure of fruits and vegetables to ozone can slow the sporulation of fungi harmful to citrus fruits, especially Geotrichum and Pennicilium.



**Treated** **Untreated**

**Figure 6.** Comparison between the states of ozone-treated and untreated food products after 24 days

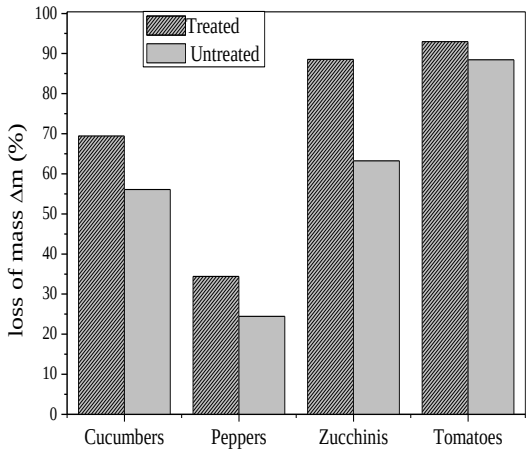



**Figure 7.** Mass loss of the food products after 24 days of storage

Moreover, the loss of mass after 24 days of storage has been analyzed; the obtained results are plotted in (Fig.7) which represents the difference between treated and untreated products in terms of mass loss. In addition to the longer food storage shelf life of the products, the loss of mass is much smaller for the products stored in the ozone-treated room, which thus represents a significant profit for the users.

Furthermore, the energy consumed by the ozone generator could be estimated by measuring the power of the ozone generator.

Lissajous figure plotted in (Fig.8) was used to analyze the consumed power. The energy consumed during one cycle of the discharge can be expressed as :

$$P = \frac{1}{nT}\int_0^{nT} v(t).i(t)dt \qquad (2)$$

Where T is the period of applied voltage, $i(t)$ is the current flowing through the discharge reactor and $v(t)$ is the applied voltage. Since the current is flowing through a measuring capacitor *C*, it can be expressed as:

$$i(t) = \frac{dq}{dt} = C\frac{dV_C}{dt} \qquad (3)$$

where $V_C$ is the voltage across C and q is the transported charge in the ozone generator, then the energy consumed per one cycle can be calculated by the following equation:

$$W = \frac{1}{nT}\int_0^{nT} v(t).C.dV_C = \frac{1}{nT}\int_0^{nT} v(t).dq(t) \qquad (4)$$

Therefore, since the energy consumed during one cycle of the discharge is equal to the enveloped area of Lissajous (equation 4), the power *P* can be calculated by multiplying this area *W* by the frequency *f*

$$P = W.f \qquad (5)$$

According to equation (5), the power consumed deduced from the Lissajous figure plotted in (Fig. 8), is equal to 40 W, corresponding to 16 W/m$^2$.

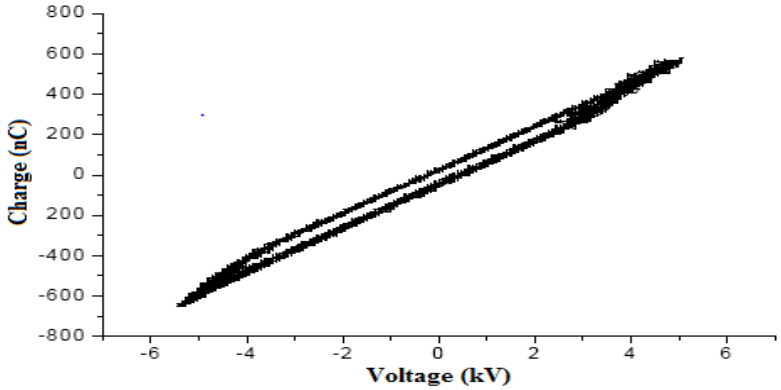



**Figure 8 .** Lissajou curve of the ozone generator

## 4. Conclusions

Although that several studies have been developed in ozone food conservation during recent years, we have noticed that there is a lack of studies on the use of this process in isolated sites not fed by conventional electric energy.

The results presented in this paper confirm the disinfection action of the ozone in food storage rooms by extending the shelf life of fruits and vegetables, by using a photovoltaic solar system that is the only solution in isolated sites.

The inverter developed using usual components such as transistor, Mosfet and a command by Arduino strongly reduces the cost of the installation.

Indeed, it has been proved through the experiments carried out, that an ozone production system supplied by a PV solar system for food storage offers a cost-effective and well-adapted solution for isolated sites.

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# PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF POLYPHENOLS EXTRACT FROM *POLYGONUM MULTIFLORUM* THUNB. ROOT

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**ABSTRACT**

The purpose of this research is to investigate the presence of alkaloids, saponins, flavonoids, anthraquinones and tannins compounds as the possible agent responsible for the medicinal activities, the antioxidant activities and antimicrobial activities from *Polygonum multiflorum* Thunb. root. The powdered root was analyzed positively for alkaloids, saponins, flavonoids, anthraquinones and tannins. In addition, they are also related to an antimicrobial activity and the presence of these constituents was helpful to apply in medical and food industry. The determination of antimicrobial activity of *Polygonum multiflorum* Thunb. root extracts against gram-negative *Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (ATCC 13076), gram-positive: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 11774), *Listeria monocytogenes* (CIP 74908), fungi: *Fusarium equiseti*, *Aspergillus niger* and *Trichoderma asperellum* were investigated by the paper disc diffusion method for antibiotic susceptibility testing and minimum inhibitory concentration (MIC) evaluation of dryness extract. The results showed that the dryness extract can inhibit one gram-positive bacteria (*Staphylococcus aureus*, MIC = 200 mg/mL), one gram-negative bacteria (*Salmonella enteritidis*, MIC = 400 mg/mL) and one fungus (*Trichoderma asperellum*, MIC = 100 mg/mL); it's not take effectively on *Escherichia coli*, *Bacillus subtilis*, *Listeria monocytogenes*, *Fusarium equiseti* and *Aspergillus niger*.

## 1. Introduction

Currently, there are a large number of herbal plants whose importance science has not been explored. All over the world, most of plants have used as the richest source of raw materials for traditional as well as modern medicine. *Polygonum multiflorum* Thunb. is one of the most popular traditional herbal plants of Vietnamese and is a main ingredient in many prescriptions during a thousand year. It was cooked with many food such as chicken, black bean (Zhou *et al*., 2010) in order to eat or use as drug to cure many diseases like tonic tension (Lim *et al*., 2014), anti-aging effects (Lin *et al*., 2008), antioxidant activity (Wang *et al*., 2008) and certain forms of cancer (Hung *et al*., 2004).

The ethnomedical uses of *Polygonum multiflorum* Thunb. that has been recorded in many provinces in Asia such as China, Korean, Japan and Vietnam. Some the scientists discovered more than 100 chemical bioactive compounds from this plant, and the major components that consisted of stilbenes,

phospholipids, quinones, flavonoids and others (Lin *et al*., 2015).

However, the content and bio-activity of these components depend on many factors such as climate, soil, harvesting season, gene, storage condition and the different extraction methods. Therefore, the determination of presence of these components is quite important in this research. Until now, many studies have demonstrated that parts of this plant contain biologically active compounds such as phenolic compounds, saponins, alkaloids, etc. They are useful in food technology or drug industry, especially root and hairy root of *Polygonum multiflorum* Thunb. Phenolic compounds in hairy root can inhibit *Staphylococcus aureus*, *Escherichia coli*, *Fusarium oxysporum* and *Aspergillus niger* (Thiruvengadam *et al.*, 2014) while phenolic compounds in root have the high antioxidant capacity (Le *and* Nguyen, 2015). There are many researches that extracted polyphenols from *Polygonum multiflorum* Thunb. root but until now there are no reports on phytochemical screening and antimicrobial activities on extract of *Polygonum multiflorum* Thunb. root. Therefore, the current research was undertaken to determine some bioactive compounds and antibacterial activities of acetone extract of *Polygonum multiflorum* Thunb. root.

## 2. Materials and methods

### 2.1. Plant collection

*Polygonum multiflorum* Thunb. roots were harvested from Cao Bang province (Vietnam) and the clean roots were sliced and dried at 60°C until < 12% moisture level was reached. The slices were ground into a fine powder (< 0.5 mm) and vacuum-packed.

### 2.2. Organisms collection

Antibacterial activity and minimum inhibitory concentration (MIC) were determined against three gram-positive bacteria as *Bacillus subtilis* (ATCC 11774), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (CIP 74908), two gram-negative bacteria as *Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (ATCC 13076) and three fungi as *Fusarium equiseti*, *Aspergillus niger* and *Trichoderma asperellum* (They were kindly provided by Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh city).

### 2.3. Acetone extraction

The powdered root was extracted by microwave-assisted extraction (MAE) with aqueous acetone 57.35%, the ratio of materials/solvent is 1/39.98 (w/v), an extraction time of 289 seconds and microwave power of 127 W (Le *and* Nguyen, 2015). The extracts were filtered by using Whatman filter paper No. 4 and evaporated under vacuum conditions in a water bath at 45°C. After that, the residue was freeze-dried during 7 hours at -20$^{o}$C, < 1 mbar and dryness extract stored at 4$^{o}$C prior to use.

### 2.4. Phytochemical analysis

#### *2.4.1. Identification of flavonoids*

Ferric chloride test: Three drops of solution of 5% $FeCl_3$ was added the extract. The formation of greenish-black color indicates the presence of phenolic nucleus (Sofowora, 1993).

Sodium hydroxide test: The extract was added about 2 mL of 10% NaOH solution, yellow solution indicates the presence of flavonoids which adds on adding dilute hydrochloric acid that becomes colorless (Evans, 2002).

#### *2.4.2. Identification of tannins*

Ferric chloride test: Three drops of solution of 5% $FeCl_3$ were added the extract, production of a blue or greenish-black color that changes to olive green as more $FeCl_3$ 5% is added to indicate the presence of tannins (Evan, 2002).

Gelatin test: Few drops of 10% gelatin solution were added to the extract. Formation of a precipitate indicates the presence of tannins.

Lead sub-acetate test: Few drops of 10% lead sub acetate solution were added to the extract. Formation of a colored precipitate indicates the presence of tannins (Evan, 2002).

### *2.4.3. Identification of anthraquinones*

Borntrager's test: The 2 mL extract was added to 5 mL chloroform in the test tube and shaken for a few minutes. The mixture was shaken with equal volume of 10% ammonia solution. After shook this mixture, there is the presence of free anthraquinones by layering such as violet, pink or red (Evan, 2002).

### *2.4.4. Identification of alkaloids*

A small extract (2 mL) was mixed with 20 mL of 5% sulphuric acid in 50% ethanol. The mixture was cooled. Two drops of concentrated ammonia solution was added into the solution, then the equal volume of chloroform was also added and shook gently to allow the separation of the individual layers. Chloroform in the lower layer is removed. The ammoniacal layer was added drop by drop by the Dragendorff's reagent. The solution appears the reddish-brown that precipitated to indicate the presence of alkaloids (Evans, 2002).

### *2.4.5. Identification of saponins*

Frothing test: The extract was placed in a test tube and added to 10 mL of distilled water; shook vigorously for 30 seconds then let keep for 30 minutes and observe. The formation of foam indicates the presence of saponins (Sofowora, 1993).

Haemolysis test: Few drops of an animal blood was added to the extract (prepared in normal saline) by a syringe and mixed gently by inverting the tube and allowed to keep for 15 minutes. The settling down of the red blood cells denotes the presence of saponins (Yusuf *et al.*, 2014).

## 2.5. Color evaluation

Color parameters were measured on extract. Values were recorded as lightness $L^*$ (ranging from 0 to 100 corresponding to black to white), $a^*$: Red shade (if the value is positive), green shade (if the value is negative) and $b^*$: Yellow shade (if the value is positive), blue shade (if the value is negative).

## 2.6. Determination of total polyphenol content (TPC) and antioxidant capacity (AC) of extract

The TPC in the extract was slightly modified and determined by the Folin-Ciocalteu colorimetric method (Siddiqua *et al.*, 2010). The results were based on a standard curve obtained with gallic acid. TPC was expressed as mg of gallic acid equivalent per gram of dry weight (mg GAE/g DW).

The AC of the extract was determined by DPPH assay which was adapted from Soto *et al.* (2014) and Chmelová *et al.* (2015), it was slightly modified. Trolox was used as the standard. AC was expressed in TEAC (Trolox equivalent antioxidant capacity) determined as µmol of Trolox per gram of dry weight (µmol TE/g DW).

## 2.7. Determination of antimicrobial activity and minimum inhibitory concentration (MIC) evaluation

The minimum inhibitory concentration (MIC) evaluation was determined by the paper disc diffusion method for antibiotic susceptibility testing according to Kirby-Bauer test (Bauer *et al.*, 1966). The sterile paper discs of 6 mm diameter were prepared that using various concentrations of dryness extract of powdered root (25, 50, 100, 200, 400, 800 and 1600 mg/mL); gentamicin (10 µg/disc) and ketoconazole (50 µg/disc) were used as positive controls to compare the antibacterial activity and antifungal activity, respectively; 5% dimethylsulfoxide (DMSO) was used as negative control. Firstly, 0.1 mL of bacteria suspension (0.5 McFarland standard, approximately $1.5\times10^8$ cfu/mL) and 0.1 mL of fungus suspension (approximately $0.4\times10^4$ – $5\times10^4$ cfu/mL) were spread on the surface of the Mueller-Hinton agar media for bacterial strains and Potato dextrose agar media for fungal strains by sterile hockey stick, respectively. Then, sterile paper discs were impregnated with 20 µL of each of extracts. The dishes were incubated during 24 hours at 37°C for bacterial strains and 72 hours at 30°C

for fungal strains. After that, the zones of inhibition were expressed in mm, as the diameters of clear zones around the discs.

### 2.8. Data analysis

Experimental results were analyzed by the one-way analysis of variance (ANOVA) method and significant differences among the means from triplicate analyses at ($p<0.05$) were determined by Fisher's least significant difference (LSD) procedure using Statgraphics software (Centurion XV). The values obtained were expressed in the form of a mean±standard deviation (SD).

## 3. Results and discussions

### 3.1. Identification of bioactive compounds

Phytochemical analysis of the powder of *Polygonum multiflorum* Thunb. root was successfully carried out, acetone was found to be a good solvent system for the extraction of the bioactive compounds of this plant. The powdered root was tested positive for alkaloids, tannins, anthraquinones, saponins and flavonoids (Table 1). These results agreed with the literature review on the plant which showed these compounds to be presented (Lin *et al.*, 2015).

Tannins were polyphenols which exist popularity in plant and were divided two types: condensed tannins and hydrolyzed tannins. Determining the presence of tannins in *Polygonum multiflorum* Thunb. root extract has many methods such as $FeCl_3$ test, gelatin test and lead sub-acetate in this research. Tannins play is an important role in food technology and human health because of their functions like they were antioxidant, anti-aging, anti-inflammatory, anticarcinogenic, etc. (Atanassova *and* Chiristova-Bagdassarian, 2009). Besides that, flavonoids were determined in *Polygonum multiflorum* Thunb. extract through reaction with NaOH solution to special-yellow. However, it is quite difficult to determine specific flavonoid due to various flavonoids, which can react with NaOH solution to create yellow color as flavone, isoflavone, flavanone, chalcone, etc. (Nguyễn, 2007). A group that is quite important compound is free anthraquinones which was also discovered in extract through red color in the aqueous layer above after adding chloroform and ammoniac solution. This bioactive compound is essential important ingredient and is anti-cancer, anti-bacterial, anti-inflammatory (Barnard *et al.*, 1992; Srinivas *et al.*, 2003) and enhance repairs the nucleotide in human's cells (Chang *et al.*, 1999).

**Table 1.** Phytochemical constituents of *Polygonum multiflorum* Thunb. root extract

| No. | Phytoconstituents | *Polygonum multiflorum* Thunb. root extract |
|---|---|---|
| 1 | **Tannins** | |
| | a. $FeCl_3$ test | + |
| | b. Gelatin test | + |
| | c. Lead sub-acetate test | + |
| 2 | **Saponins** | |
| | a. Frothing test | + |
| | b. Haemolysis test | + |
| 3 | **Anthraquinones** | |
| | Borntrager's test | + |
| 4 | **Flavonoids** | |
| | a. $FeCl_3$ test | + |
| | b. NaOH test | + |
| 5 | **Alkaloids** | |
| | Dragendorff's test | + |

*+: Present*

In addition, saponins also exit in *Polygonum multiflorum* Thunb. extract. This compound can be found in plant, especially medical plants such as *Paullinia pinnata* Linn (Yusuf *et al.*, 2014), *Acorus calamus* and *Lantana camara* (Mamta *and* Jyoti, 2012), etc. Saponins can dissolve easily in water and decrease the surface tension on solution to create more bubble honeycomb structure. This is a sample method to determine the presence of saponins in extract (Nguyễn, 2007). Saponins can make the settling down of the red blood cells when mixed with animal blood (Yusuf *et al.*, 2014). However, we can not determine exactly specific saponins (triterpenoid or steroid) in this case. The extract of *Polygonum multiflorum* Thunb. also has the presence of alkaloids which is bioactive and heterocyclic chemical compound. It contains nitrogen and may some pharmacological, medicinal or ecological activity in many cases (Aniszewski, 1994). Alkaloids can be found in animal and plant such as tea, coffee, pepper… These alkaloids are highly reactive substances with biological activity even in low doses (Aniszewski, 2007).

In general, *Polygonum multiflorum* Thunb. was precious herbal plant. Root had many bioactive compounds, it was very necessary in food and medical technology. These compounds are the main key in the medicinal value and the data can help us choose this valuable plant with greater quantity of medical and food industry.

### 3.2. Determination of total polyphenol content (TPC) and antioxidant capacity (AC) of extract

After extraction process, the TPC and AC values of extract achieve 47.53±0.79 mg GAE/g DW and 334.07±3.04 μmol TE/g DW, respectively. TPC and AC of samples from MAE method were higher than samples from China which were extracted by decoction method with deionized water as solvent (33.91±0.62 mg GAE/g DW; 257.9±3.7 μmol TE/g DW) and maceration method with 50% ethanol as solvent (40.42±0.63 mg GAE/g DW; 256.7±0.7 μmol TE/g DW) (Li *et al.*, 2007). The results show that the difference of extraction methods, land, gender, analyzation method, etc which cause the changes about TPC and AC values.

### 3.3. Identification of physicochemical characteristic of dryness extract

The moisture of extracts after freeze-drying was approximately 3.03±0.5% and stored at 4$^{o}$C. Storage conditions were quite advantageous to maintain the content and the activity of bioactive compounds such as low moisture, cold environment and less oxygen. It can be avoided the oxidization reaction and denature bioactive compounds.

The yield of dryness extract achieves 6.26±0.47%, this result is higher than recent study of Đái *et al*. (2015) (3.55%, extract from leaf and trunk of *Streptocaulon juventas* Merr.). The cause of difference was various materials, extraction method (solvent, material/solvent ratio, temperature and time extraction)… especially dewater method. Freeze-drying method can be removed completely free-water from material and a remaining part of fixed-water. Moreover, the sublimation of water does not affect significantly bioactive compounds at low temperature and low pressure (Nireesha *et al.*, 2013). This was proved that TPC and AC of dryness extract did not change significantly after freeze-drying process, TPC and AC values reached 47.15±0.88 mg GAE/g DW and 337.43±9.24 μmol TE/g DW, respectively.

Besides that, color of material also changed clearly. First, *Polygonum multiflorum* Thunb. extract has light yellow brown color. Then, the dryness extract has dark-brown color after freeze-drying. Meanwhile, $L^*$, $a^*$ and $b^*$ values also change strongly; $L^*$ value decreases rapidly from 58.98±0.16 to 36.35±0.19; $a^*$ value increases slowly from 8.21±0.02 to 12.77±0.1 and $b^*$ value decrease extremely from 20.81±0.06 to 9.86±0.27. Initial color converts into dark color and turns brown-red shade. This may be explained that the loss of

water will increase the concentration of extract which lead cause to change color and the extract that was oxidized because of long freeze-drying time.

### 3.4. Antimicrobial activity and minimum inhibitory concentration (MIC) evaluation

Antimicrobial activity of *Polygonum multiflorum* Thunb. dryness extract was studied at various concentration (25, 50, 100, 200, 400, 800 and 1600 mg/mL) against five strains of pathogenic bacteria including two gram-negative bacteria (*Escherichia coli* – ATCC 25922, *Salmonella enteritidis* – ATCC 13076), three gram-positive bacteria (*Staphylococcus aureus* – ATCC 25923, *Bacillus subtilis* – ATCC 11774, *Listeria monocytogenes* – CIP 74908) and three fungus *Fusarium equiseti*, *Aspergillus niger* and *Trichoderma asperellum*.

DMSO is special solvent which can dissolve polar and nonpolar compounds, as the negative control that do not affect to antimicrobial result. This solvent was used widely in many studies about antibacterial method, for instance Nitiema *et al.* (2012) who used coumarin and quercetin against *E. coli* and *Salmonella*, Su *et al.* (2015) that used extract of *Polygonum cuspidatum* against *S. aureus.* In addition, DMSO is also the negative control for antifungals experiment such as *A. flavus, A. niger, C. albicans, etc.* (Usharani *et al.*, 2015) or *C. gloeosporioides* and *C. capsici* on chili (Chutrakul *et al.*, 2013).

**Table 2.** Antibacterial activities of extract of *Polygonum multiflorum* Thunb. root

| Bacterial strains | Zone of inhibition (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DMSO (5%) | Gentamycin (10 µg/disc) | Concentration of dryness extract (mg/mL) | | | | | |
| | | | 50 | 100 | 200 | 400 | 800 | 1600 |
| *E. coli* | - | $19.67\pm0.58^{d}$ | - | - | - | - | - | - |
| *S. enteritidis* | - | $14\pm1.00^{Bb}$ | - | - | - | $8.67\pm0.58^{Aa}$ | $10\pm1.00^{Aa}$ | $12.67\pm0.58^{B}$ |
| *S. aureus* | - | $15.67\pm1.15^{Cc}$ | - | - | $8.33\pm1.53^{A}$ | $10.33\pm1.15^{ABa}$ | $11.67\pm0.58^{Ba}$ | NT |
| *B. subtilis* | - | $15.33\pm0.58^{bc}$ | - | - | - | - | - | - |
| *L. monocytogenes* | - | $11.33\pm0.58^{a}$ | - | - | - | - | - | - |

-: not detect, NT: not tested.
Various lowercase letters in the same column denote significant difference ($p<0.05$).
Various uppercase letters in the same row denote significant difference ($p<0.05$).

**Table 3.** Antifungal activities of extract of *Polygonum multiflorum* Thunb. root

| Fungal strains | Zone of inhibition (mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | DMSO (5%) | Ketoconazole (50 µg/disc) | Concentration of dryness extract (mg/mL) | | | | | | |
| | | | 25 | 50 | 100 | 200 | 400 | 800 | 1600 |
| *A. niger* | - | $14\pm1^{a}$ | NT | NT | - | - | - | - | - |
| *F. equiseti* | - | $25\pm1^{b}$ | NT | NT | - | - | - | - | - |
| *T. asperellum* | - | $26.67\pm2.08^{Cb}$ | - | - | $8.33\pm0.58^{A}$ | $8.67\pm0,58^{A}$ | $11\pm1^{B}$ | NT | NT |

-: not detect, NT: not tested.
Various lowercase letters in the same column denote significant difference ($p<0.05$).
Various uppercase letters in the same row denote significant difference ($p<0.05$).

Besides, gentamicin used also as positive control and had clearly effective in five of bacteria. Concentration of gentamicin was quite low (10 µg/disc) but antibacterial capacity for each bacteria strain was very different. Inhibitor zone of positive control listed in susceptible order: *L. monocytogenes* < *S. enteritidis* < *B. subtilis* < *S. aureus* < *E. coli* (Figure 1). Gentamicin is antibiotic which belongs to aminoglycosides group, can prohibit protein synthesis and destroys bacteria cell membrane system. Gentamicin diffuse inside the periplasmic space and the transport across the cytoplasmic membrane requires metabolic energy from the electron transport system in an oxygen-dependent process. Then, gentamicin binds quickly bacteria ribosome and inhibitory protein synthesis process. It decreases the

exactly of information RNA that results in the wrong combination of amino acid in polypeptide chain of bacteria (Zembower *et al.*, 1998).

The antifungal mechanisms of ketoconazole that cause increased membrane permeability, inhibition of uptake of precursors of RNA, DNA and synthesis of peroxidative and oxidative enzymes. In addition, ketoconazole derivatives inhibit the biosynthesis of ergosterol, the main sterol in the membranes of fungi. The demethylation from lanosterol to ergosterol was blocked by ketoconazole. This lead to be leaky membranes, permeability changes and fungi were easily inhibited (Van-Tyle, 1984). The results show that ketoconazole (50 μg/disc) inhibited these fungi and the inhibitor zone of positive control listed in susceptible order: *A. niger* < *F. equiseti* < *T. asperellum* (Figure 2).

Table 2 and 3 show that dryness extract of *Polygonum multiflorum* Thunb. has antimicrobial activity against a gram-positive bacteria (*S. aureus*, MIC of 200 mg/mL and inhibition zone of 8.3±1.53 mm), a gram-negative bacteria (*S. enteritidis*, MIC of 400 mg/mL and inhibition zone 8.67±0.58 mm) and a fungus (*T. asperellum*, MIC of 100 mg/mL and inhibition zone 8.33±0.58 mm). Inhibition zones of *S. enteritidis*, *S. aureus* and *T. asperellum* were "sensitive" (inhibition zone from 8 to 14 mm), this results were evaluated similar with antimicrobial level of some essential oils (Ponce *et al.*, 2003). Antimicrobial effect increases with the increase of concentration of dryness extract and depends on many different factors, for instance the presence of flavonoids in plant is advantageous against bacterial pathogen and fungi because flavonoids can destroy the cell membrane (Ikigai *et al.*, 1993), reduce permeability of membrane (Tsuchiya *and* Iinuma, 2000) and inhibit the nucleic acid synthesis (Mori *et al.*, 1987). Each type of flavonoids can inhibit microorganism by many different pathways.

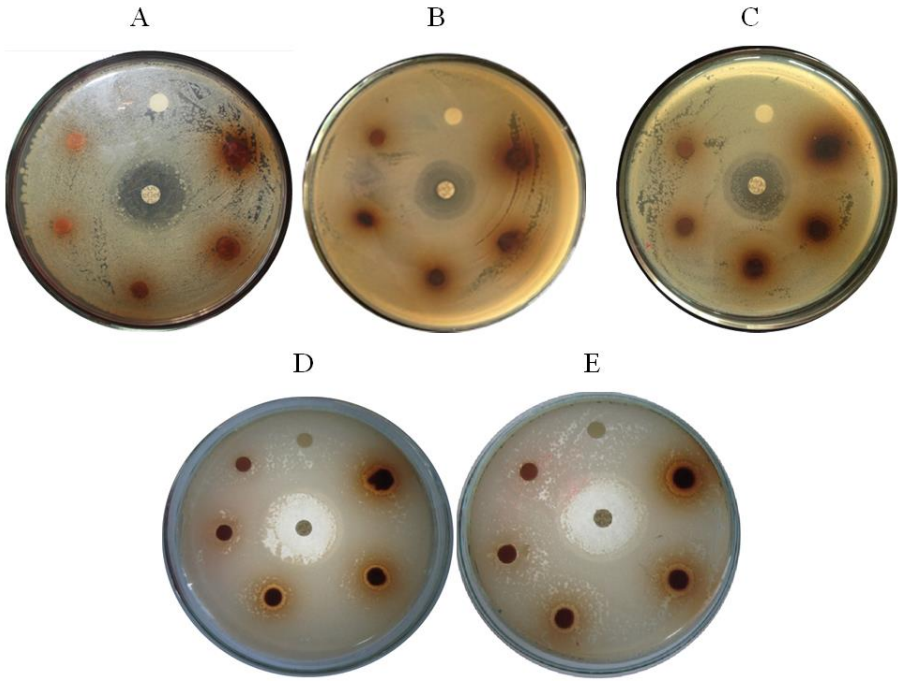


**Figure 1.** Inhibition zones for *E. coli* (A), *B. subtilis* (B), *L. monocytogenes* (C), *S. enteritidis* (D) and *S. aureus* (E).

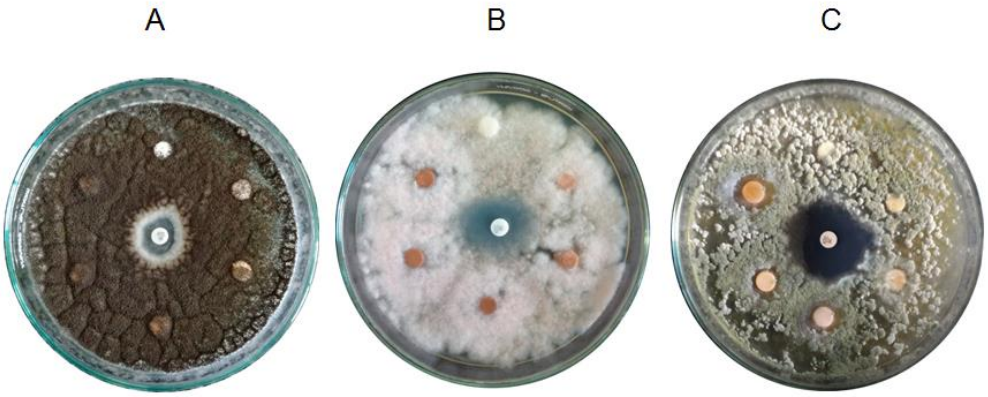


**Figure 2.** Inhibition zones for *A. niger* (A), *F. equiseti* (B), *T. asperellum* (C)

According to Scalbert (1991) and Rebecca *et al.* (2009), there are three hypothesis that might explain the antimicrobial mechanism of tannins on microorganism: inhibition of enzyme activity by complexing with substrates of bacteria and fungi; direct action of tannins on the microorganism metabolism, through the inhibition of oxidative phosphorylation; a mechanism involving the complexation of tannins with metabolic ions, decreasing the availability of essential ions to the metabolism of the microorganisms. Besides, tannins in extract also can inhibit gram-negative and gram-positive bacterial, especially condensed tannins (Zarin *et al.*, 2016). However, hydrolyzed tannins can inhibit some specific bacteria such as *S. aureus* (Lim *et al.*, 2006). In addition, some studies show that these compounds almost don't affect resistance of yeast and fungi because they have thick walled structure and high chitin content (Madigan *and* Martinko, 2006) but this results show that *T. asperellum* was inhibited by the present of tannins in extract. Besides, tannins in *Stryphnodendron adstringens* extract also inhibited *Candida albicans* (Santos *et al.*, 2009).

According to Lin *et al.* (2015), anthraquinones in *Polygonum multiflorum* Thunb. root include rhein, emodin, aloe-emodin, physcion, chrysophanol, etc. After entering the cell membrane, emodin will bind to DNA and destroy bacteria (Lu *et al.*, 2011). Furthermore, rhein, emodin and aloe-emodin also inhibit respiration of some bacteria, especially *S. aureus* (Chen *et al.*, 1963) and interfere in redox of enzyme NADH dehydrogenase in bacteria (Zhang *and* Chen, 1986). The present of anthraquinones in extract could be due to the leaks in the cell wall or perhaps some alteration in the membrane permeability, resulting in the loss of the cytoplasm and fungus were inhibited (Phongpaichit *et al.*, 2004).

Some bioactive compounds except polyphenols can inhibit microorganism such as saponins and alkaloids. These compounds also present in *Polygonum multiflorum* Thunb. root extract. Alkaloids can inhibit the synthesis of DNA, RNA and cellular respiration of microorganism (Aniszewski, 2007). There are many studies show that the bacteria which was inhibited by alkaloids in plant, for instance like *L. monocytogenes* and *S. typhimurium* that were inhibited by alkaloid extract of *Pangium edule* (Chye *and* Sim, 2009); *E. coli, S. aureus, B. cereus, S. carmonum,* etc. were sensitive with alkaloids extract of *Sida acuta* (Karou *et al.*, 2005). Alkaloids from *Lupinus luteus* L. extract can inhibit many fungi such as *Rhizoctonia solani, Phoma exigua,* etc. (Sas-Piotrowska *et al.*, 1996).

The presence of saponins also contributed significantly to the antibacterial activity of the extract. However, saponins affect strongly gram-positive bacteria more than gram-negative bacteria and fungi (Soetan *et al.*, 2006). Antimicrobial activity depends on structure of aglycon of saponins. The possible antimicrobial mechanism of saponins was due

to the reduced glucose utilization efficiency in microorganisms, then affecting their growth and proliferation, reducing the activity of key enzymes in physiological metabolism and suppressing the synthesis of relevant proteins, and finally executing the antibacterial effect (Yu *et al.*, 2013). Some bacteria was inhibited by saponins extract from plant, for instance *E. coli, S. aureus, K. pneumonia, B. subtilis* and *P. aeruginosa* were inhibited by saponins extract from *Anabasis articulate* (Maatalah *et al.*, 2012). Saponins from *Gymnema sylvestre* and *Eclipta prostrata* leaves extract inhibited *A. flavus, A. niger, A. fumigatus* (Gopiesh-Khanna *and* Kannabiran, 2008).

The obtained result shows that there are many bioactive compounds in the dryness extract. They can inhibit microorganism by many different mechanisms. Hence, combination of these compounds has the anti-microorganism effect better than single compound.

## 4. Conclusions

The presence of bioactive compounds was determined. The powdered root had many bioactive compounds such as alkaloids, saponins, tannins, flavonoids and anthraquinones. These components were quite precious compound to apply to medical technology and food industry. In addition, acetone dryness extract from *Polygonum multiflorum* Thunb. root proved to be effective against both one gram-positive bacteria (*Staphylococcus aureus*) at MIC of 200 mg/mL, one gram-negative bacteria (*Salmonella enteritidis*) at MIC of 400 mg/mL and a fungus (*Trichoderma asperellum*) at MIC of 100 mg/mL. Therefore, the result shows that this plant had antioxidant and antimicrobial potentials. It may be used as alternative natural sources applicable to medicine, agriculture and food products.

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# ANTIOXIDANT AND ANTIMICROBIAL EFFECTS OF ETHANOL EXTRACT OF *SCROPHULARIA STRIATA* PLANT ON QUALITY OF FILLET CHICKEN DURING REFRIGERATOR STORAGE

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**ABSTRACT**

Oxidative and pathogenic problems are some of the main challenges for fillet chicken meat products recently; however, now these products have been recommended as alternatives for processed red meat products. In this study, antibacterial and antioxidant effects of *Scrophularia striata* ethanol extract on fillet chicken during the 7-day refrigerator storage time have been investigated. Fillet chicken samples treated with 1, 3 and 5% *Scrophularia striata* ethanol extract were analyzed for microbiological and chemical analysis during the storage time. The antioxidant activity and antibacterial effect as well as against Staph. aureus of fillet chicken samples with 5% *Scrophularia striata* ethanol extract were demonstrated higher than other concentrations. Consequently, *Scrophularia striata* plant ethanol extract were recommended as antioxidant and antibacterial agents for treatment of fillet chicken as well as this result was corresponded with sensory evaluation of treated samples.

## 1. Introduction

Consumption of poultry meat products has been enhanced recently all over the world. These products are preferred by consumers instead of red meats for many nutritional reasons. Poultry meat products are reach in some nutritional values including vitamin E (tocopherols), minerals, essential amino acids, ascorbic acid and poly unsaturated fatty acids (PUFA); However, increasing level of PUFA in poultry meat leads vitamin E content of these products to be decreased (Valsta, Tapanainen, & Männistö, 2005). Tocopherol components have protection rule of PUFA from oxidative activities. Chicken meat is one of the most important poultry products with similar nutritional value but lower selenium and vitamin E content (Owens, 2010).

The quality properties of poultry meat products include texture, flavor, functionality and microbial quality influencing satisfactory of consumers. Flavor characteristics in simple and complex poultry products such as sausages, marinated, cooked and frozen fillets require special consideration as well as sensory properties influenced easily by oxidative reactions occurred usually in these products. Unsaturated fatty acids content in poultry meat products induce strong oxidative activity to produce oxidation final products leading to flavor undesirability (Fletcher, 2002). Also, unsaturated fatty acid oxidation process releases some carcinogenic components making oxidative stress to form DNA damages as well as safety condition for consumption of these products is not

acceptable. Another important factor in safety consumption of poultry meat products are microbiological properties as well as pathogens and toxins released by them (Kim, Warner, & Rosenvold, 2014). Some high risk pathogens are considerable in poultry meat simple and complex products including Listeria monocytogenes, Escherichia coli, Staphylococcus aureus, Salmonella and Campylobacter spp. These high risk pathogens enter the poultry meat products via initial and cross contamination. Activities against prevalence of these pathogens through poultry meat product manufacturing and distribution leads to assure safety consumption (Kozačinski, Hadžiosmanović, & Zdolec, 2006). Another aspect of microbiological characteristics of poultry meat products is the shelf life problem. Microbial counts of these products indicate the shelf life domain as well as higher microbial counting implying less shelf life (Mataragas, Skandamis, & Drosinos, 2008).

Application of plant extract as an antioxidant and antibacterial agent in food products have been suggested by many researchers recently. Extract of plants are obtained in aqueous and ethanol solutions naming aqueous and ethanol extracts, respectively. Phytochemical compounds including organic acids, essential oils, flavones, alkaloids, phenolics, tanins and terpenoids in plant extracts contribute to antibacterial and antioxidant properties. plant extracts are formulated with food and packaging materials for preservative purposes in food technology (Hsieh, Mau, & Huang, 2001). These extracts released from different parts of plant such as root, flower, leaves, seeds, fruits, bulb, herb and husk. Many plant extracts have suggested by researchers for employing in food technology for antimicrobial and antioxidant effects with family names including Arceae, Bixaceae, Lamiaceae, Liliaceae, Meliaceae, Myrtaceae, Poaceae and Rutaceae (Katalinic, Milos, Kulisic, & Jukic, 2006). Babuskin et al. in the year 2014 employed spice extract for shelf life extension raw chicken meat. They observed suitable antioxidant and antibacterial effect of spice extract for effective preservation of chicken meat. Phenolic constituents of spice extract were considered as strong antimicrobial and antioxidant agents to enhance microbial and oxidative quality of raw chicken meat during the storage preservation (Babuskin et al., 2014).

Scrophularia striata plant is classified to the family scrophulariaceae. The genus Scrophularia is the most important one between 300 species of scrophulariaceae family and usually grows in Turkey, Azerbaijan and Iran areas. There many phytochemical compounds detected in extract of this plant with expanded domain of functionality. Most important components known from Scrophularia extract with considerable antioxidant and antibacterial activity consist of quercetine, isorhamnetin-3-o-rutinoside, nepitrin, cinnamic acid and phenyl propanoid glycoside (Monsef-Esfahani, Hajiaghaee, Shahverdi, Khorramizadeh, & Amini, 2010). Sharafati-chaleshtori and Rafieian-kopaie (2014) observed antibacterial effect of Scrophularia striata plant extract against E. coli in vitro condition (Sharafati-Chaleshtori & Rafieian-Kopaei, 2014). Also, Bahrami and Ali in the year 2010, investigated the antimicrobial effect of Scrophularia striata plant ethanol extract and they found strong activity against S. aureus. Ethanolic extract of Scrophularia striata have been suggested as antioxidant and antibacterial additive for food and packaging formulation by many researchers (Bahrami & Ali, 2010). The aim of this study was to investigate effects of antibacterial and antioxidant activity of Scrophularia striata ethanol extract on

chemical and microbial quality properties of fillet chicken during the refrigerator storage.

## 2. Materials and methods

### 2.1. Bacterial culture

Lyophilized culture of Staphylococcus aureus NCTC 29213, a laboratory isolate was purchased from and confirmed by Pasteur institute of Paris, France. Before inoculation the bacterial culture to the samples, the cultures were incubation in nutrient broth then in saline overnight at 35 ˚C to reach 105 CFU mL-1 then isolated subcultures before the start of experiments. Obtained suspensions were diluted by saline solution as required in advance.

### 2.2. Sample preparation

Scrophularia striata plant was purchased from local market in Zanjan, Iran. Purchased plants were cleaned and washed then dried by sun. Fruits and leaves of the plant were separated and milled to produce powder form. For production of ethanol extract of plant, 100 grams of the powder form of Striata was added to 500 mL of 80% ethanol then mixed for 24 h at ambient temperature. After filtration through Whatman paper, ethanol was separated from mix extract by evaporation under vacuum condition at 50 ˚C (Bahrami & Ali, 2010). Finally, stoke obtained extract was preserved at 4 ˚C in dark place until sample preparation. Chicken fillet were purchased for experiments from local markets in Zanjan, Iran. 50-gram pieces of chicken fillet samples inoculated with Staphylococcus aureus were suspended in 0, 1, 3 and 5% Scrophularia striata plant ethanol extract for 5 min then stored at 4 ˚C then samples were taken at days 0, 2, 5 and 7 of refrigerator storage time for chemical and microbiological analysis. All treatments have been implemented in triplicate.

### 2.3. Chemical analysis

Lipid peroxidation give rise to produce Thiobarbituric Acid Reactive Substrate (TBARS) determined as an indicator of oxidation process of fat using the method suggested by Kannat et al. (2010). 4 grams of each samples were mixed with 16 mL of 5% trichloroacetic acid and BHT then filtered through Whatman paper. The filtrate in equal amount added to 0.02 M TBA, heated in a water bath for 30 min then cooled to the room temperature for absorbance measurement at 532 nm. The amount of TBARS was expressed as mg malonaldehyde per kg of fillet chicken meat. Peroxide value of oil extracted from fillet chicken meat samples was measured that is suggested by AOAC-965.33 (International, 2005). 3-5 grams of extracted oil was added to acetate-chloroform (2:3) with 0.5 mL potassium iodide. After shaking and addition of 30 mL distilled water, solution was titrated with 0.01 N sodium thiosulfate until the blue color of starch indicator was observed. The peroxide value of samples was calculated by the following equation (Eq. (1)):

$$PV = (V \times N \times 1000)/W \quad (1)$$

While PV is peroxide value, V is the volume of sodium thiosulfate and W is the weight of extracted oil. For measuring pH vale, 10 grams of fillet chicken meat sample mixed with 90 mL of distilled water then the pH value was determined by a digital pH-meter instrument (Metrohm 744, Netherland). All measurements were conducted in triplicate (International, 2005).

### 2.4. Microbial analysis

Effect of Scrophularia striata plant ethanol extract on microbiological attributes of fillet chicken meat during refrigerator storage time including days 0, 2, 5 and 7 was evaluated. Microbiological analysis of

treated samples was carried out according to agar based plate counting method. Ten grams of each samples were taken and placed into the sterile plastic container then diluted with 90 mL buffered water. Diluted sample was homogenized in a stomacher (Stomacher 400, England) for 1 min then prepared in suitable decimal dilutions. For counting total aerobic mesophilic bacteria diluted samples was incubated into plate count agar (PCA, Merck, Germany) for 48 h at 37 ˚C. Total aerobic psychrophilic bacteria were measured by incubation of samples in plate count agar (PCA, Merck, Germany) for 7 days at 7 ˚C. Staphylococcus aureus was counted using Bird Parker Agar (BPA, Merck, Germany) incubated for 24 h at 37 ˚C. All measurements in plate counting were carried out in triplicate (Muhammad et al., 2013).

### 2.5. Sensory evaluation

Each sample was grilled and cooked ready for sensory evaluation by panelists. A panel team consist of 30 persons semi-experienced in evaluation of meat products sensory attributes was used. Each panelist previously had become familiar with sensory properties of cooked fillet chicken meat. All panelists were asked to evaluate taste, color, texture and total acceptability of samples. Sensory characteristics were evaluated using 5 point hedonics scale (0-5). The scale points include: very poor (1), poor (2), acceptable (3), good (4) and very good (5) (Feng et al., 2016).

### 2.6. Statistical procedure

All analysis and measurements in this study were carried out in triplicate. Analysis of Variance (ANOVA) was used to determine significantly ($p<0.05$) between treatments and the contrast between means (Duncan`s multiple range test for chemical, microbiological and sensory analysis) were used to assess the differences between the variables. Statistical analyses were conducted using SPSS ver. 22 for windows (Chicago, USA).

## 3. Results and discussions

### 3.1.Chemical analysis

Fig. 1 provides pH value changes during the 7-day storage time of fillet chicken samples different ethanol extract of Scrophularia striata plant. As can be seen in Fig. 1, pH values of all samples increase gradually during the storage time final pH value of sample with 5% extract (the highest concentration) is the lowest one (pH = 6.13) in comparison with the other similar treated samples with lower plant extract. Variations of PV through refrigerator storage time are demonstrated in Fig. 2. As it can be observed from Fig. 2, PV increasing of samples with higher plant extract were less than others; consequently, samples with 5% plant extract demonstrated the lowest PV (1.91 meq O2/Kg lipid) after 7 days indicating the best oxidative condition. Oxidative reactions of fatty acids give rise to enhancement of pH value and PV due to creating some compounds explained these phenomena. These observations concluded that Scrophularia striata plant extract has the antioxidant effect and prevents oxidative reactions specially in food systems. Azadmehr et al. (2009), Monsef-esfahani et al. (2010), Ghoran et al. (2012) and Mahboubi et al. (2013) reported antioxidant activity of Scrophularia striata plant aqueous and ethanol extracts (Azadmehr et al., 2009; Ghoran, Safavi, Meighani, & Ebrahimi, 2012; Mahboubi, Kazempour, & Nazar, 2013; Monsef-Esfahani et al., 2010) . Pasdaran et al. (2012) also showed high antioxidant activity of essential oil of Scrophularia striata plant (Pasdaran, Delazar, Nazemiyeh, Nahar, & Sarker, 2012).

TBA value of treated samples with different Scrophularia striata plant ethanol extract are

shown in Fig. 3. Considering the Fig. 3, it can be concluded that TBA value of samples with high concentration of plant extract have lower enhancement than others; however, this result had been seen for pH value and PV previously. Lipid oxidation can be monitored by TBARs test as higher TBA value indicates better oxidative condition and low levels of oxidation. Antioxidant activity and characteristic of Scrophularia striata plant ethanol extract prevent oxidation in treated fillet chicken samples. Phenolic compounds, flavonoids, cinnamic acid and phenyl propanoid in Scrophularia striata plant lead to higher antioxidant activity. Azadmehr et al. in the year 2013, also showed the antioxidant and neuroprotective activity of Scrophularia striata plant extract usable in medical and food technology (Azadmehr et al., 2009).

### 3.2. Microbiological properties

As can be seen from Figs 4 and 5, total aerobic bacteria and total psychrophilic bacteria increase gradually during the 7-day refrigerator storage time in fillet chicken treated samples with Scrophularia striata ethanol extract. The final point, after 7 days storage, was observed the lowest counting for both total anaerobic bacteria and total psychrophilic bacteria with 5% Scrophularia striata ethanol extract treatment. As reported by Mahboubi et al. (2013), Scrophularia striata extract have significant antimicrobial effect on gram positive and negative bacteria in comparison with antibiotic treatment. However, methanol and ethanol extract of Scrophularia striata plant observed more effective for antibacterial characteristic. The phenolic and flavonoid compound in Scrophularia striata extract were recommended to be the cause of antibacterial effect of this plant extract (Mahboubi et al., 2013). Abbasi et al. (2007) also observed antimicrobial properties of Scrophularia striata extract on various range of bacteria (Abbasi, Azizi Jalilian, Abdi, & Saifmanesh, 2007). Antibacterial effect of Scrophularia striata extract against E. coli also was demonstrated by Sharafati-chaleshtori et al. (2014) (Sharafati-Chaleshtori & Rafieian-Kopaei, 2014).

Fig. 6 provides effect of Scrophularia striata ethanol extract on Staph. aureus inoculated into the fillet chicken samples during the storage time. Trends in figure indicated significant increase in samples treated with 1% Scrophularia striata ethanol extract and control; but, decrease then increase observed for 3 and 5% extract treatments overall leads to considering no change in Staph. aureus counting in samples after 7 days storage time significantly. Abbasi et al. in the year (2007) showed antibacterial effect of Scrophularia striata extract against Staph. aureus. They reported phenolic and flavonoid compound in Scrophularia striata plant extract are causes of antibacterial effect against Staph. aureus in this extract. Also, this plant extract is recommended useful against pathogenic bacteria in food product formulation (Abbasi et al., 2007). Bahrami and Ali in the year 2010 also demonstrated antibacterial effect of Scrophularia striata leaves ethanol extract against Staph. aureus significantly (Bahrami & Ali, 2010).

### 3.3. Sensory evaluation

The results of sensory evaluation including color, odor, flavor, texture and total acceptability of fillet chicken treated with 1, 3 and 5% Scrophularia striata ethanol extract were demonstrated in Fig. 7. As it can be seen in data from figure 7; color, odor, texture, flavor and total acceptability were observed high score for sensory evaluation. As a result, Scrophularia striata ethanol extract without any sensory preventive effect can be used as antibacterial and antioxidant effect in fillet chicken products.

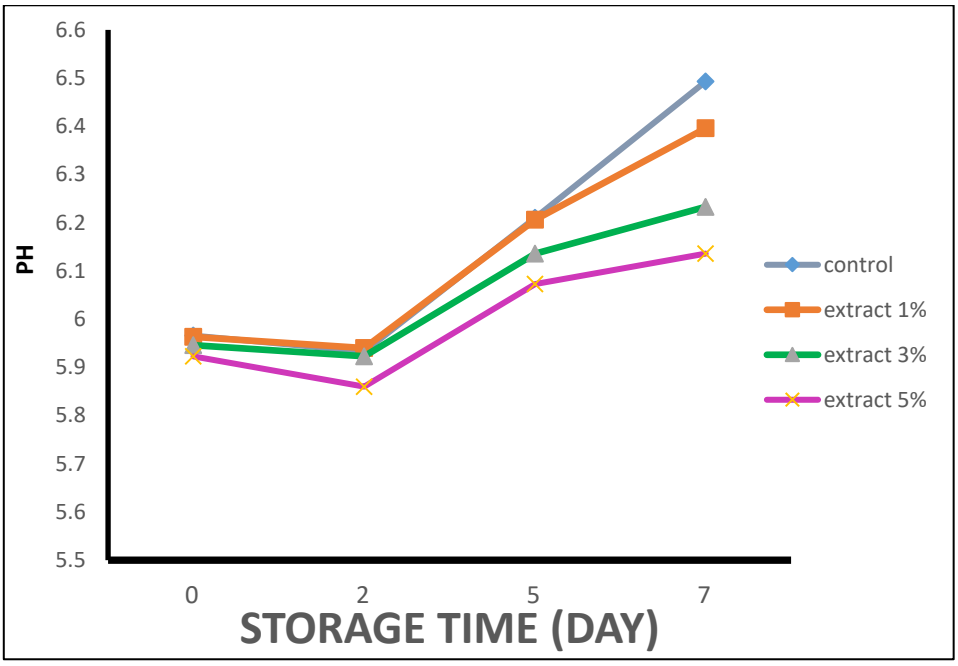


**Figure 1.** pH changes during the refrigerator storage time of fillet chicken samples treated with Scrophularia striata ethanol extract

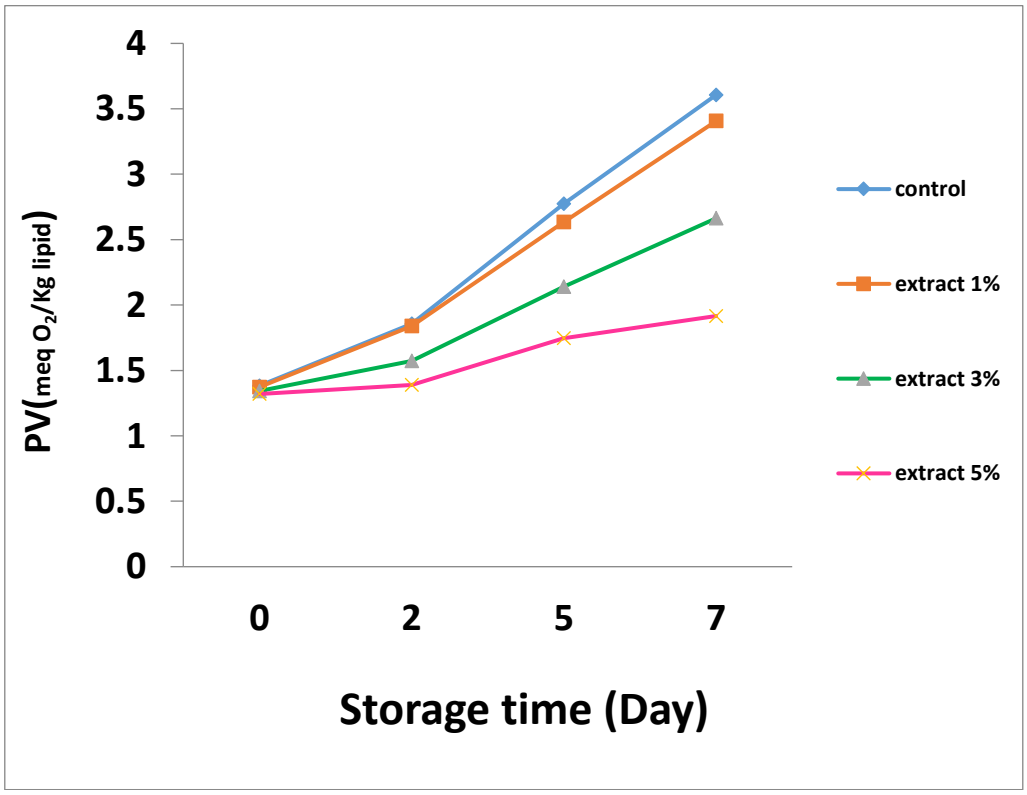


**Figure 2.** PV variations during the refrigerator storage time of fillet chicken samples treated with Scrophularia striata ethanol extract

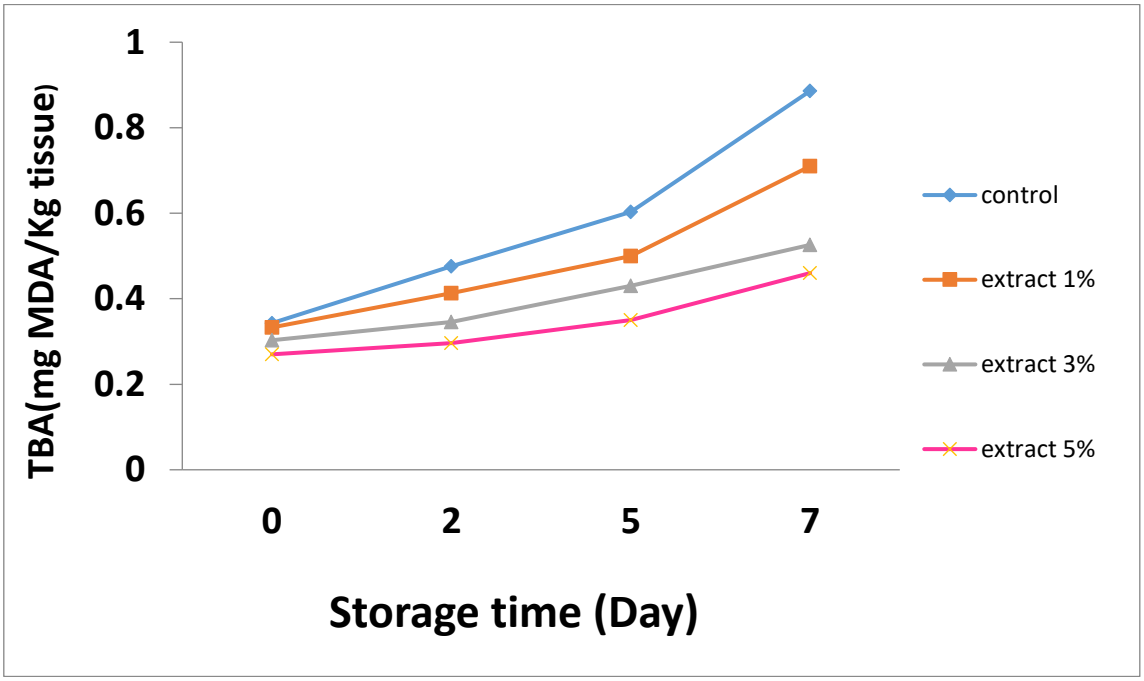


**Figure 3.**TBA value during the refrigerator storage time of fillet chicken samples treated with Scrophularia striata ethanol extract

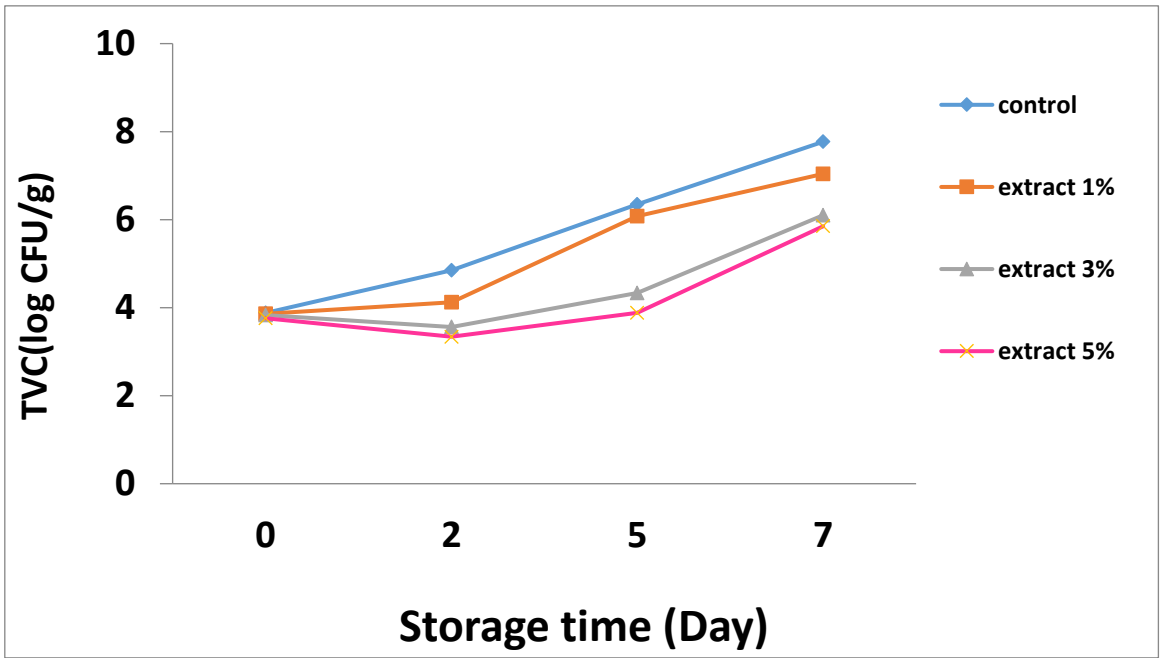


**Figure 4.** Total aerobic count during the refrigerator storage time of fillet chicken samples treated with Scrophularia striata ethanol extract

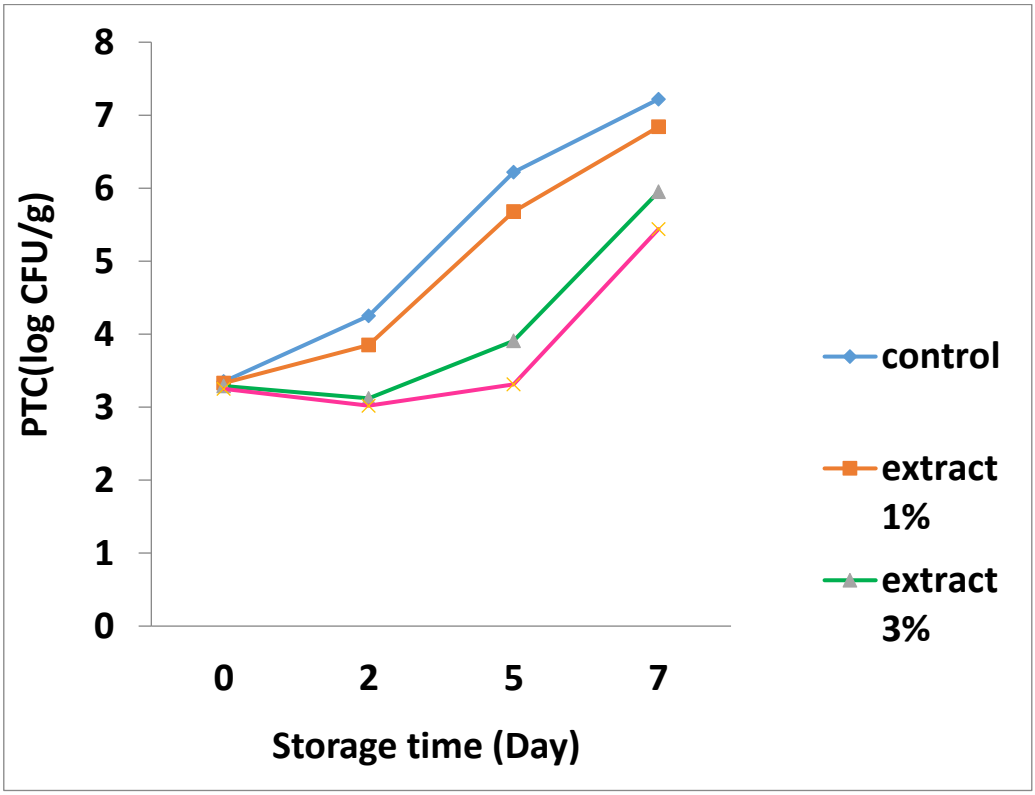


**Figure 5.** Total psychrophilic count during the refrigerator storage time of fillet chicken samples treated with Scrophularia striata ethanol extract

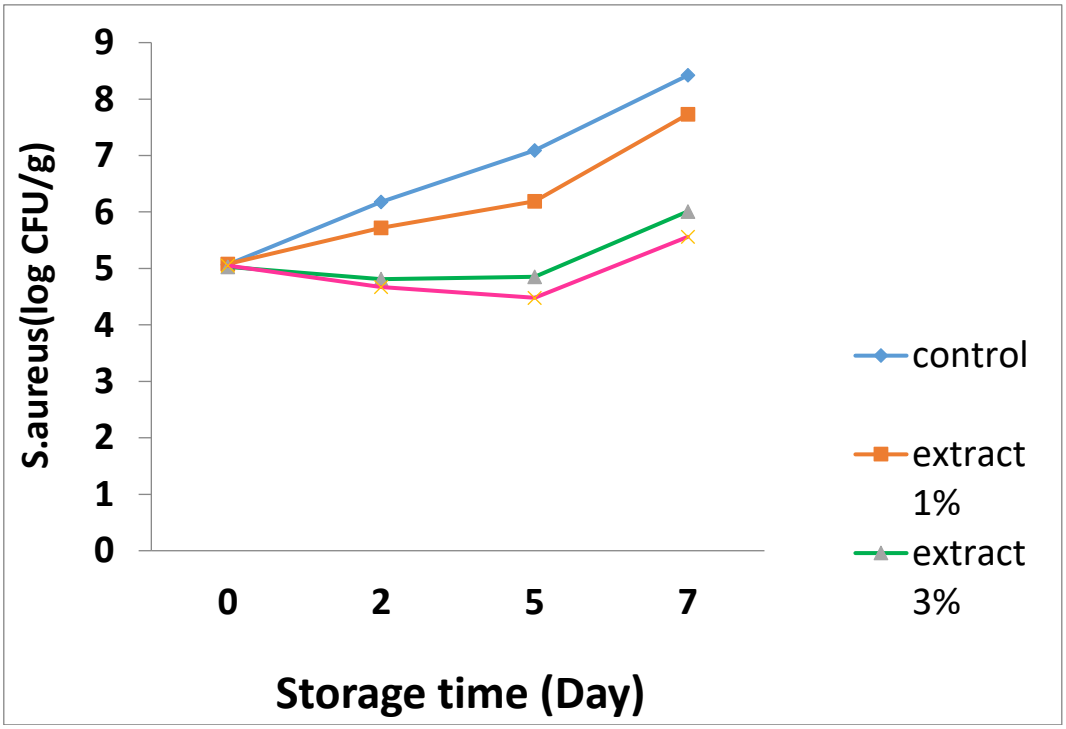


**Figure 6.**Total count of Staph. aureus during the refrigerator storage time of fillet chicken samples treated with Scrophularia striata ethanol extract

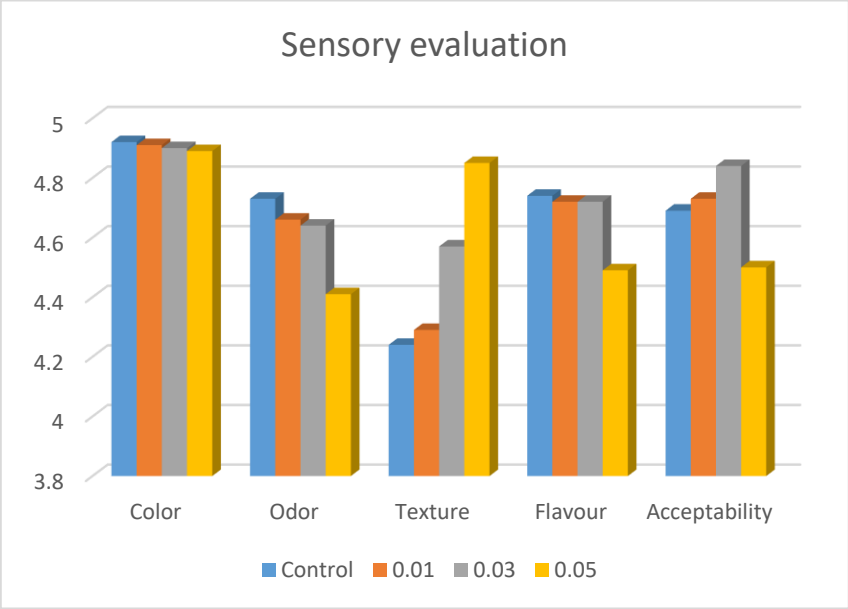


**Figure7.** Sensory evaluation of fillet chicken samples treated with Scrophularia striata ethanol extract after 7-day refrigerator storage time

## 4. Conclusions

As conclusion, antibacterial and antioxidant effects of Scrophularia striata ethanol extract on fillet chicken during the 7-day refrigerator storage time were observed significantly. Fillet chicken samples treated with 1, 3 and 5% Scrophularia striata ethanol extract were analyzed for microbiological and chemical analysis during the storage time. The antioxidant activity and antibacterial effect against Staph. aureus of fillet chicken samples with 5% Scrophularia striata ethanol extract were demonstrated higher than other concentrations. As a result, in this study Scrophularia striata plant ethanol extract were recommended as antioxidant and antibacterial agents for treatment of fillet chicken as this was corresponded with sensory evaluation of treated samples.

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# OPTIMIZATION OF PROCESS PARAMETERS FOR MICROWAVE ASSISTED UV STERILIZATION SYSTEM FOR ORANGE JUICE

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**ABSTRACT**

A microwave assisted ultraviolet light sterilization system (MWUV) was developed to study the synergistic effect in the sterilization of orange juice. This study used Response surface methodology (RSM) based on Box-Behnken design to get the optimum sterilization condition of MWUV and to analyse its effect on viable bacterial count and biochemical properties. Three independent variables; microwave power (200–500 W), flow rate (120–200 mL/min) and treatment time (0–20 sec) were taken for this study. The optimized processing parameters such as total plate count for bacterial load (1.26 log CFU/mL), total phenols (641 mg GAE/L), L* (57.63), a* (6.37), b* (53.81) and Vitamin C (264.2 mg/L) were found at the microwave power (500 W), flow rate (166 mL/min) and treatment time (9.51 sec). The fresh untreated sample was taken as control. The results showed MWUV could be a fast and effective method for sterilization of orange juice and other liquid foods without negotiating the quality of the sample.

## 1.Introduction

Liquid foods such as milk, vegetables and fruits juices are globally accepted as nutritious. The Dietary Guidelines for Americans 2010 recommend us to make one-half of our plate fruits and vegetables. Diets high in such liquid foods are widely recommended for their health-promoting properties. From the early time, fruits and vegetables play a key role in our daily diets because of their richness in vitamins (mainly C and A), minerals, natural antioxidants and abundant flavonoids. Moreover, they are considered as a rich source of dietary fibre. Nowadays in the highly demanding global market, the consumers demand for fruit juices are becoming more because of their health consciousness; therefore, is high requirement for new value added and properly processed products to meet consumer demand for convenience, nutrition, and health.

Citrus fruits and their products are widely consumed because of their health beneficiaries due to high content of vitamin C and other biologically active compounds such as polyphenols, flavonoid, limonoid, carotenoid and fibre. Orange (*Citrus cinensis*) belongs to the genus citrus of the *Rutaceae* family. These juices are consumed as non-alcoholic beverages and their demand in the market continues to rise due to increasing awareness of their health benefits. On the other hand, there is still a problem and risk of pathogenic infections that are related with the consumption of these juices. Different processing and preservations techniques are adopted to reduce this health risk. However, conventional heat (thermal) and prolong microwave sterilization

processing of these citrus juices may destroy the bioactive compounds present in them, thus reducing beneficial health effects (Plaza et al., 2006). To reduce the unwanted effects (loss of nutrients and natural qualities) of the thermal processing methods, other alternative methods that are capable of microbial inactivation can be used. For this, non-thermal methods for processing juices such as high pressure processing (HPP), pulse electric field (PEF), ultrasonic waves, ultraviolet radiation (UV) and their combinations to get a synergistic effect are emerging technologies that are becoming more popular these days (Aronsson et al., 2001; Mertens, 1992; Toepfl et al., 2007). Therefore, to ensure the safety for consumption, to maintain natural fresh quality of the juices, there is an utmost need for an alternative treatment method for longer storage life, microwave assisted UV treatment may also be a positive solution of these problems.

Many researchers had showed the effects of microwave treatment for processing and preservation on food products (Hayet et al., 2010; Polydera et al., 2003). Microwave heating works superior to conventional heating over slow thermal diffusion that results in slow cooking and burning of outside layer which is not found in microwave heating. The microwave pasteurization and sterilization for liquid food retain better quality due to lower thermal exposure i.e. require less processing time to inactivate enzymes and most heat resistant microbes. Sterilization by conventional methods may not be possible without altering the bio-chemical properties of orange juice (Handwerk & Coleman, 1998; Lee & Nagy, 1998). Combination of both microwave and UV reduced the microbial load exponentially in waste water treatment (Mishra et al., 2010).

For processing of food materials by UV light, a specific range of wavelength is used which varies from 100 to 400 nm and is classified as UV-A (320–400 nm), UV-B (280–320), and UV-C (200–280 nm). Short-wavelength UV radiation UV-C (200-280 nm) is regarded as the germicidal region fatal to most of microorganisms (Bintsis et al., 2000; Sizer & Balasubramaniam, 1999). Since 1980s, disinfection of water by chlorination process has been replaced by UV radiation in many countries (Gibbs, 2000). UV irradiation treatment in foods has been approved and recommended by the US Food and Drug Administration (FDA) and US Department of Agriculture (USDA). It may be a suitable method for preserving various liquid foods such as milk, fruit juices and other beverages. During the UV treatment, there is no known toxic and substantial non-toxic by-products are formed that will harm the human health. But it may not be considered as a standalone method for complete sterilization of liquid foods because it works effectively on the surface and limited to bulkiness, organic solutes, suspended particles and colour of the juice (Koutchma, 2008; Falguera et al., 2011). UV radiation can be generated by an electrodeless lamp powered by the electromagnetic waves generated by microwaves at the frequency of 2450 MHz. When microorganisms are exposed to UV radiation, cellular DNA absorbs the energy by purines and pyrimidine bases, and adjacent thymine molecules links together that damages the cellular metabolism and kills the microorganisms (Reisz et al., 2014; Billmeyer, 1997; Giese, 1997).

Therefore, the primary aim of this study is to optimize the processing parameters involved in the microwave assisted UV sterilization system of liquid foods. The system was developed to combine both the microwave and UV radiations and to study the synergistic effect on microbial load and quality parameters of orange juice by

developing a lab scale microwave assisted ultraviolet sterilization (MWUV) system.

## 2. Materials and methods

### 2.1. Materials

Fresh orange fruit was procured from Rourkela market, Odisha, India. The raw samples were immediately taken to the laboratory for cleaning, juice extraction and then for microbial and quality analysis. A household microwave oven of frequency 2450 MHz (1200 Watts output) was used for the treatment in which mercury gas filled electrodeless lamps (Albatross UV, Part No. 558432, H-Type, USA) with dimensions (length - 152.4 mm, diameter - 9 mm) and power output of 300 watts per inch were added as per design required for treatment.

### 2.2. Extraction of fruit juices

The quality parameters like shape, size, colour and scratch-free were taken into account for choosing the fruits. The selected ones were sorted and washed thoroughly under tape water to get remove the surface microbes and contaminations. The peel was removed by using a stainless steel knife and the rinds and seeds were taken out from the juicy pulp manually to avoid bitterness of the extracted juice. Then the pulps were blended using a grinder (Bajaj Mixer, India) and filtered with the help of muslin cloth. The filtrate was immediately packed and kept in sterilized airtight glass bottles at 5 °C for further experimentations.

### 2.3. Optimization of sterilization process

The liquid samples were treated to microwave alone as well as microwave assisted ultrasound treatments according to different power or time combinations given by experimental design at a particular treatment time. Microwave irradiation power, A (200-500 W), flow rate, B (120-200 mL/min) and treatment time, C (0-20 sec) were taken as independent variables. Untreated raw sample was taken as a control. All the experiments were done in triplicate to get precise results. After the treatments, the treated samples were immediately taken for microbial and biochemical analysis.

### 2.4. Experimental setup

Box–Behnken Design was used to optimise the MWUV treatment parameters viz. microwave power, flow rate and treatment time with respect to the responses such as microbial count (total plate count), and biochemical properties; colour (L*, a*, b* values), total phenols and vitamin C. Analysis of data and model creation were executed by using the Design Expert Software (Version 10.0.7.0, Stat-Ease, Inc., Minneapolis, MN 55413) for optimisation of variables processing parameters. Table 1 shows the range and centre point values of the three independent variables (microwave power, flow rate and treatment time). Through the design software, a total of 17 (seventeen) experiments at 5 (five) replications at the centre point were designed. A second order polynomial equation can be used to show the effect of the dependent variables on the independent variables and also acts as a function of independent variables (Equation 1).

$$Y = b_o + \sum b_1 X_1 + \sum b_{11} X_1^2 + \sum b_{12} X_1 X_2 \quad (1)$$

where Y is the experimental responses; $X_1$ and $X_2$ the levels of the variables; $b_0$ is the constant; $b_1$ the linear coefficient; $b_{11}$ the quadratic term; and $b_{12}$ the coefficient of the interaction terms. Analysis of variance (ANOVA), regression analysis and surface plotting (Figures 1 to 4) were performed to establish optimum condition for microwave assisted UV treatment on fruit juices. Three-dimensional response surface plots were achieved by changing the variables; keeping one variable constant at the centre point and changing the remaining two variables in the experimental range.

**Table 1.** Independent variables and their level used for central composite design

| Independent variables | Units | Level | | |
|---|---|---|---|---|
| | | -1 | 0 | +1 |
| Microwave irradiation power (A) | W | 200 | 350 | 500 |
| Flow rate (B) | mL/min | 120 | 160 | 200 |
| Treatment time (C) | sec | 0 | 10 | 20 |

### 2.5. Microbial analysis

Total plate count is the most common method used to quantify the total number of viable bacteria in foods, water and all processed foods. Bacterial cells present in food form colonies when nutrient medium is provided, which can be counted to find the number of cells in the sample. The results are expressed as the number of Colony Forming Units (CFU) per ml of the sample (CFU/ml). Nutrient agar was taken as a nutrient medium which supported growth of different types of bacteria. Molten and autoclaved nutrient agar was transformed to Petri plates to form agar plates. On solidifying of agar, inoculation of bacteria was done from the diluted samples. The plates were kept in incubator at 37 ± 1 °C (Anderson et al., 2011; Das et al., 2015). Bacterial colonies were counted in digital colony counter, after 24 hours of incubation.

### 2.6. Biochemical analysis

Both microwave assisted UV treated and row juices were subjected for the biochemical analysis. Colour values (L*, a*, b*), total phenolic content and vitamin C content were determined as response parameters for biochemical properties of the fruit juice. A Colorimeter (HunterLab Color Flex EZ spectrophotometer, USA) was used to measure the colour values that gave precise of values of L* - lightness / darkness, a* - redness / greenness, and b* - yellowness / blueness of the samples. Determination of vitamin C was done by titration method explained by Mazumdar and Majumder using 2, 6 – dichloroindophenol (DCIP) dye solution (Mazumdar B.C. & Majumder K., 2003). The amount of total phenolic compounds was found out by using the Folin–Ciocalteu method; using gallic acid as standard (Abdullakasim et al., 2007). Absorbance values of the samples were measured at 765 nm wavelength using a Spectrophotometer (Perkin Elmer Lambda 25-UV/VIS, USA). The total phenolic compounds of the samples were expressed as milligrams per liter Gallic acid equivalents (mg GAE/L).

## 3. Results and discussions

Tables 2 and 3 show the Box-Behnken design matrix and dependent variables with their respective coefficients of determination ($R^2$), coefficient of variance (C.V.) and standard deviation (Std. Dev.) respectively. Statistical analysis indicated that the proposed model was adequate, possessing no significant lack of fit and with satisfactory values of the $R^2$ for the total phenol, colour values (L*, a*, b*), vitamin C and total plate count. Table 4 shows the actual and predicted values of all the responses generated by Box-Behnken design. Generally, a higher value of coefficient of variances shows that difference in the mean value is high and does not satisfactorily develop an adequate response model (Ravikumar et al., 2006).

**Table 2.** Box-Behnken design matrix

| Expt. No. | Microwave assisted UV (A) | Feed rate (B) | Treatment time (C) | Total phenols (mg GAE/L) | L* | a* | b* | Vitamin C (mg/L) | Total plate count (log CFU/mL) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | -1 | -1 | 0 | 628.56 | 58.00 | 6.35 | 52.6 | 275.44 | 3.49 |
| 2 | 1 | -1 | 0 | 650.78 | 57.33 | 6.43 | 53.53 | 261.56 | 2.63 |
| 3 | -1 | 1 | 0 | 592.89 | 60.00 | 6.28 | 54.66 | 293.78 | 4.50 |
| 4 | 1 | 1 | 0 | 624.44 | 57.00 | 6.36 | 54.01 | 270.00 | 3.10 |
| 5 | -1 | 0 | -1 | 590.00 | 60.00 | 6.28 | 54.00 | 285.00 | 7.00 |
| 6 | 1 | 0 | -1 | 631.00 | 59.00 | 6.36 | 54.17 | 278.89 | 3.00 |
| 7 | -1 | 0 | 1 | 616.00 | 58.33 | 6.35 | 53.97 | 265.56 | 5.22 |
| 8 | 1 | 0 | 1 | 615.00 | 56.33 | 6.44 | 53.47 | 230.00 | 1.10 |
| 9 | 0 | -1 | -1 | 606.11 | 59.00 | 6.36 | 54.08 | 260.00 | 3.22 |
| 10 | 0 | 1 | -1 | 570.34 | 60.67 | 6.26 | 54.71 | 295.11 | 1.26 |
| 11 | 0 | -1 | 1 | 615.00 | 57.33 | 6.41 | 53.38 | 249.55 | 2.60 |
| 12 | 0 | 1 | 1 | 620.00 | 57.00 | 6.51 | 54.00 | 260.00 | 8.00 |
| 13 | 0 | 0 | 0 | 630.44 | 58.67 | 6.32 | 54.28 | 279.44 | 4.89 |
| 14 | 0 | 0 | 0 | 620.00 | 59.00 | 6.33 | 54.00 | 267.11 | 2.33 |
| 15 | 0 | 0 | 0 | 631.00 | 58.33 | 6.41 | 53.50 | 267.00 | 1.13 |
| 16 | 0 | 0 | 0 | 625.00 | 59.00 | 6.35 | 54.00 | 269.00 | 6.00 |
| 17 | 0 | 0 | 0 | 631.00 | 59.33 | 6.30 | 54.28 | 267.00 | 1.30 |

### 3.1. Total plate count

The linear terms microwave assisted UV power and treatment time were found to be significant at $p < 0.001$. As the microwave power and treatment time increases the total plate count decreases. The interaction terms between microwave assisted UV and treatment time were found to be significant at $p < 0.05$. As the combined effect of microwave power and treatment time decrease the total plate count and is shown in Figure 1. The quadratic term of treatment time is significant at $p < 0.001$. The coefficient of determination and adjusted

coefficient of determination was 96.90 and 93.11 (Table 3) and were extremely fitting the data obtained from total plate count. The reason for this better sterilization may be because of the extra stress added by the UV radiation given to the microorganisms against their growth (Koutchma, 2008; Steed et al., 2008). The results obtained from this technique of sterilization met the World Food Programme Standard.

### 3.2. Colour values (L*, a*, b*)

From the Figure 2 (c-h), it can be observed that the effect of independent process parameters on dependent variables and their responses. The linear terms of L* values i.e. microwave assisted UV and treatment time showed significant negative values at $p < 0.001$ while flow rate showed insignificant difference at $p > 0.10$. The interaction terms microwave assisted UV and flow rate; flow rate and treatment time showed significant negative values at $p < 0.001$ and $p < 0.05$ respectively. Some quadratic terms such as microwave assisted UV and flow rate showed significant negative difference at $p < 0.05$ and $p < 0.10$. The coefficient of determination and adjusted coefficient of determination found to be very high for L* values. There was a slight decreased in L* value (untreated sample - 61.82; optimized value - 57.63); Table 5. This shows the orange juice turned little bit dark due to the heating effect of microwave treatment. Similar findings were reported by different researchers (Wibowo et al., 2015; Cortés et al., 2008; Cserhalmi et al., 2006; Lee & Coates, 2003). The linear term flow rate showed significant negative difference on a* values. Also, the interaction terms between flow rate and treatment time showed significant positive difference on a* values. The data points do not fit extremely well because the coefficient of determination ($R^2$) and adjusted coefficient of determination (Adj $R^2$) were found to be low i.e. 81.82 and 58.46 respectively (Table 3). There was a slight increased in a* value with the increased in flow rate and microwave power whereas reverse was the case for treatment time. This indicates the juice became little bit reddish the treatment (Wibowo et al., 2015; Koka et al., 2004).

The linear terms microwave assisted UV and flow rate were found to be significant at $p < 0.001$ and $p < 0.10$. b* values decreased as the microwave power and flow rate increased. The interaction term between microwave assisted UV power and flow rate were found to be significant at $p < 0.05$ and the b* value decreased as this interaction term values increased. The coefficient of determination and adjusted coefficient of determination were found to be 82.56 and 60.15 respectively (Table 3). This decrease may be due to partial precipitation of unstable, suspended particles in the treated orange juice (Rivas et al., 2006; Genovese et al., 1997).

### 3.3. Vitamin C

All the linear terms i.e. microwave assisted UV power, flow rate and treatment time were found to be significant at $p < 0.001$. Vitamin C increases as the flow rate increases whereas decreases when microwave power increases Figure 3 (i and j). The reason for decrease in the vitamin C content may be because of the effect of heat generated by microwave since it is very heat sensitive (Cinquanta et al., 2010; Vikram et al., 2005). The interaction terms between microwave assisted UV power and treatment time; flow rate and treatment time were found to be significant at $p < 0.05$ and 0.10 respectively. The data fits well because the coefficient of determination ($R^2$) and adjusted coefficient of determination (Adj $R^2$) were found to be 94.23 and 86.83 respectively Table 3. The quadratic term of treatment time found to be significant at $p < 0.05$.

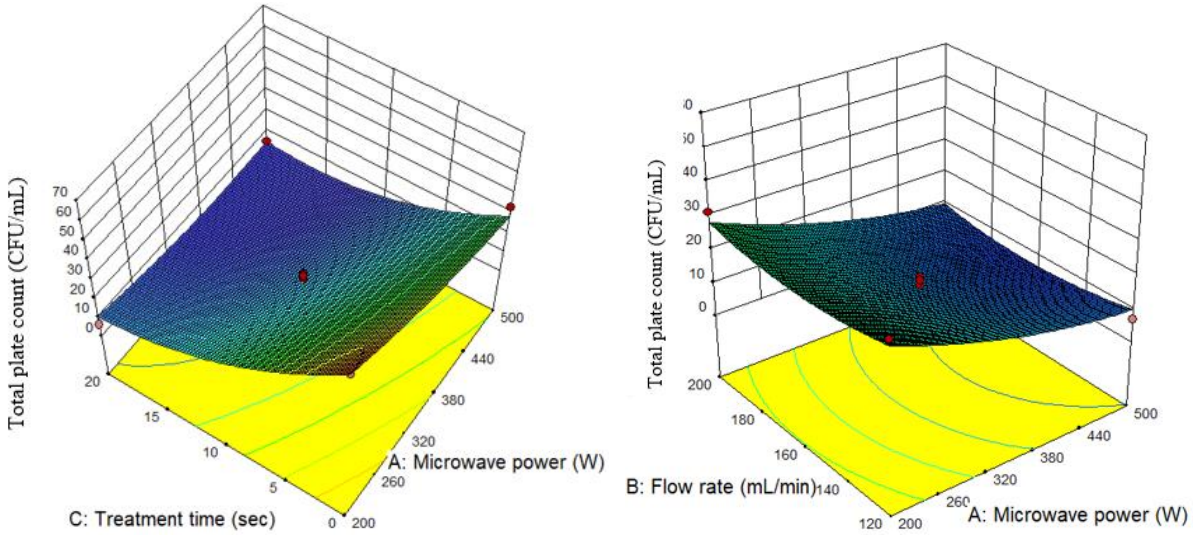
**Table 3.** Regression coefficients, standard deviation (Std. Dev.), $R^2$, and CV values for six dependents variables for the microwave assisted UV treatment

| Coefficient | Total phenols | L* | a* | b* | Vitamin C | Total Plate Count |
|---|---|---|---|---|---|---|
| Intercept | 627.49 | 58.87 | 6.340 | 53.900 | 269.91 | 12.87 |
| A | 11.72*** | -0.83*** | 0.041 | 0.0063*** | -9.92*** | -9.79*** |
| B | -11.60*** | 0.38 | -0.017*** | 0.470* | 9.04*** | -0.20 |
| C | 8.57** | -1.21*** | 0.056 | -0.270 | -14.24*** | -18.95*** |
| $A^2$ | 3.41 | -0.43** | -0.0073 | -0.230 | 1.99 | 2.60 |
| $B^2$ | -6.73 | -0.35* | 0.020 | -0.086 | 3.29 | 3.80 |
| $C^2$ | -17.90*** | -0.01 | 0.023 | 0.120 | -7.04** | 9.84*** |
| AB | 2.33 | -0.58*** | 0.001 | -0.390** | -2.47 | 0.0075 |
| AC | -10.50** | -0.25 | 0.0025 | -0.170 | -7.36** | 5.20** |
| BC | 10.19* | -0.50** | 0.050** | -0.0025 | -6.17* | -1.40 |
| Std. Dev. | 8.72 | 0.33 | 0.042 | 0.300 | 5.70 | 4.36 |
| C.V. (%) | 1.42 | 0.57 | 0.660 | 0.560 | 2.12 | 21.27 |
| $R^2$ | 90.75 | 96.67 | 81.820 | 84.17 | 94.23 | 96.90 |
| Adj $R^2$ | 78.85 | 92.39 | 58.460 | 60.15 | 86.83 | 93.11 |

*** Significant at $p < 0.001$
** Significant at $p < 0.05$
* Significant at $p < 0.10$

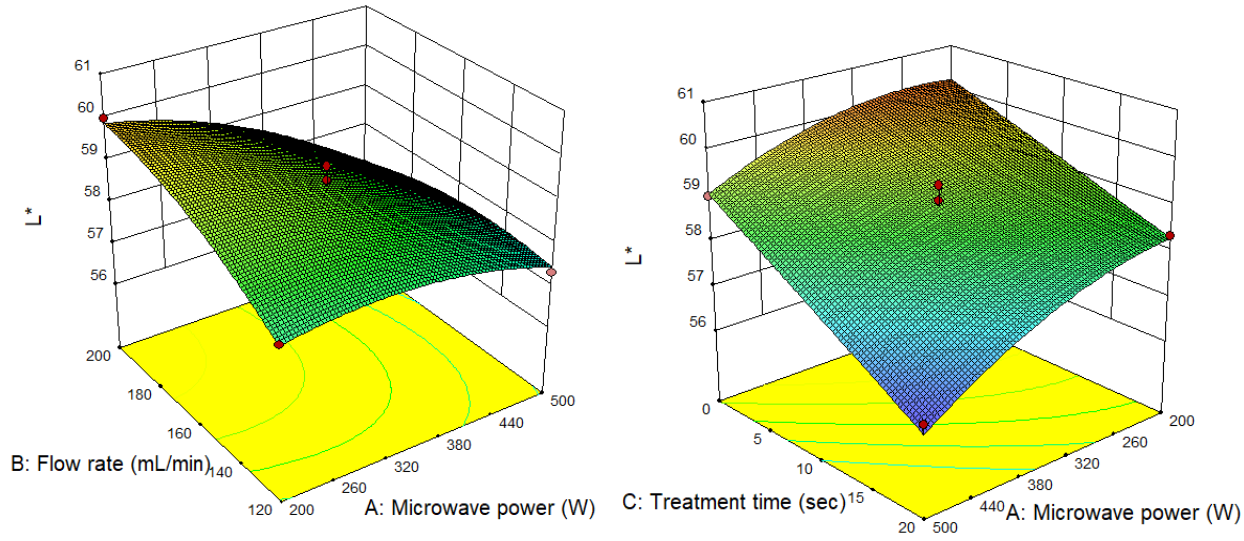
**Table 4.** Box–Behnken design: Actual and predicted values of the responses

| Expt. No. | Total phenols (mg GAE/L) | | L*-value | | a*-value | | b*-value | | Vitamin C (mg/L) | | Total plate count log(CFU/mL) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Act | Pre | Act | Pre | Act | Pre | Act | Pre | Act | Pre | Act | Pre |
| 1. | 628.56 | 626.38 | 58.00 | 57.96 | 6.35 | 6.33 | 52.60 | 52.84 | 275.44 | 273.59 | 3.49 | 1.68 |
| 2. | 650.78 | 645.15 | 57.33 | 57.45 | 6.43 | 6.41 | 53.53 | 53.62 | 261.56 | 258.71 | 2.63 | 1.52 |
| 3. | 592.89 | 598.52 | 60.00 | 59.88 | 6.28 | 6.30 | 54.66 | 54.58 | 293.78 | 296.63 | 4.50 | 4.85 |
| 4. | 624.44 | 626.62 | 57.00 | 57.04 | 6.36 | 6.38 | 54.01 | 53.77 | 270.00 | 271.85 | 3.10 | 2.89 |
| 5. | 590.00 | 582.21 | 60.00 | 60.21 | 6.28 | 6.26 | 54.00 | 54.01 | 285.00 | 281.65 | 7.00 | 7.58 |
| 6. | 631.00 | 626.65 | 59.00 | 59.04 | 6.36 | 6.34 | 54.17 | 54.33 | 278.89 | 276.55 | 3.00 | 3.27 |
| 7. | 616.00 | 620.35 | 58.33 | 58.29 | 6.35 | 6.37 | 53.97 | 53.81 | 265.56 | 267.91 | 5.22 | 4.95 |
| 8. | 615.00 | 622.79 | 56.33 | 56.12 | 6.44 | 6.46 | 53.47 | 53.46 | 230.00 | 233.35 | 1.10 | 1.76 |
| 9. | 606.11 | 616.08 | 59.00 | 58.83 | 6.36 | 6.40 | 54.08 | 53.83 | 260.00 | 265.19 | 3.22 | 3.27 |
| 10. | 570.34 | 572.50 | 60.67 | 60.59 | 6.26 | 6.26 | 54.71 | 54.79 | 295.11 | 295.61 | 1.26 | 1.66 |
| 11. | 615.00 | 612.84 | 57.33 | 57.41 | 6.41 | 6.41 | 53.38 | 53.30 | 249.55 | 249.05 | 2.60 | 1.16 |
| 12. | 620.00 | 610.03 | 57.00 | 57.17 | 6.51 | 6.47 | 54.00 | 54.25 | 260.00 | 254.81 | 8.00 | 5.95 |
| 13. | 630.44 | 627.49 | 58.67 | 58.87 | 6.32 | 6.34 | 54.28 | 54.01 | 279.44 | 269.91 | 4.89 | 3.64 |
| 14. | 620.00 | 627.49 | 59.00 | 58.87 | 6.33 | 6.34 | 54.00 | 54.01 | 267.11 | 269.91 | 2.33 | 2.82 |
| 15. | 631.00 | 627.49 | 58.33 | 58.87 | 6.41 | 6.34 | 53.50 | 54.01 | 267.00 | 269.91 | 1.13 | 2.67 |
| 16. | 625.00 | 627.49 | 59.00 | 58.87 | 6.35 | 6.34 | 54.00 | 54.01 | 269.00 | 269.91 | 6.00 | 5.82 |
| 17. | 631.00 | 627.49 | 59.33 | 58.87 | 6.30 | 6.34 | 54.28 | 54.01 | 267.00 | 269.91 | 1.30 | 2.22 |

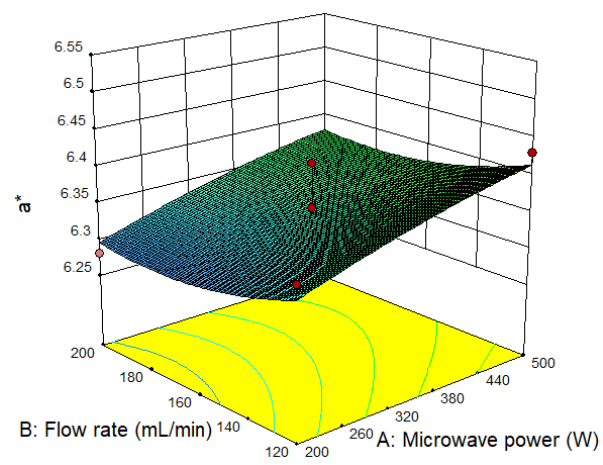



**(a)** **(b)**

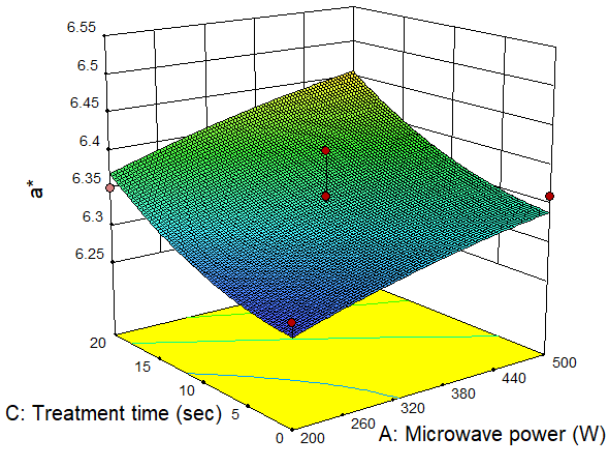
**Figure 1 (a, b).** Response surface plots (3D) for total plate count as function of microwave power, flow rate and treatment time.



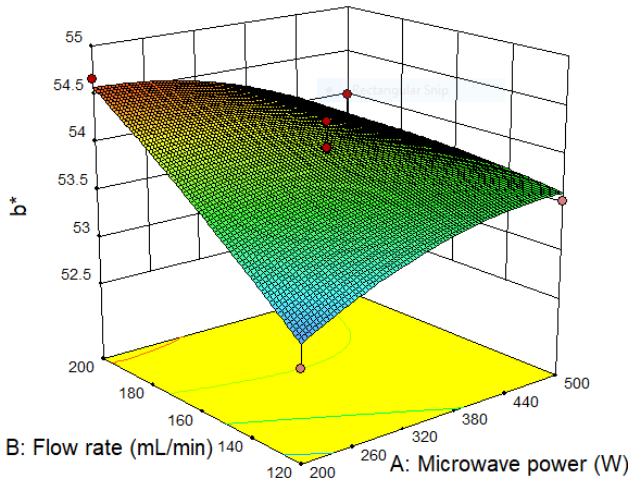

**(c)** **(d)**



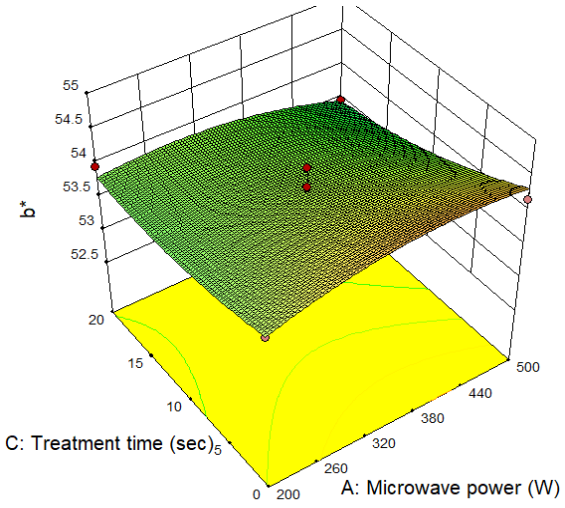

**(e)**




**(f)**

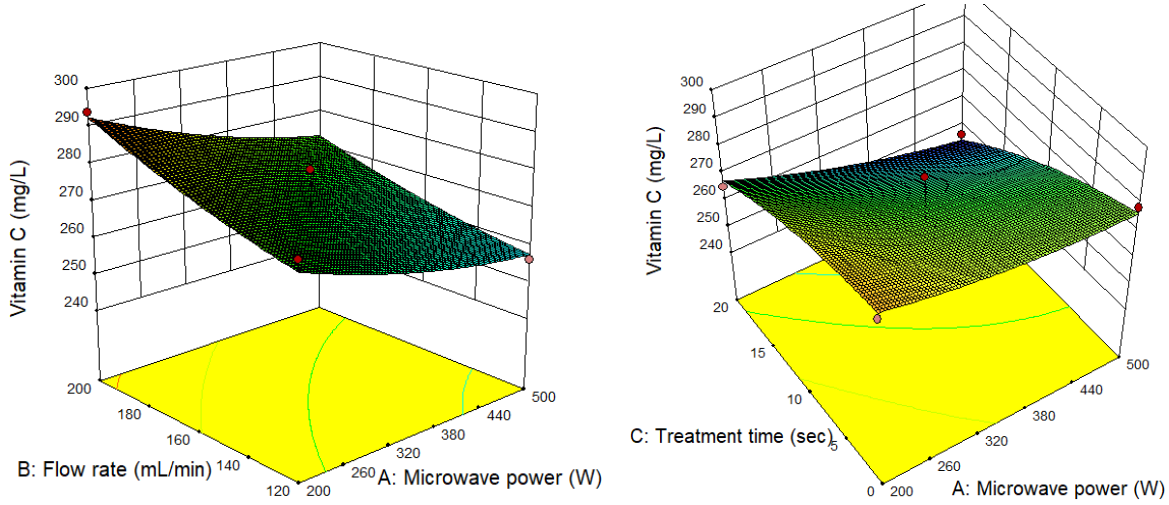



**(g)**



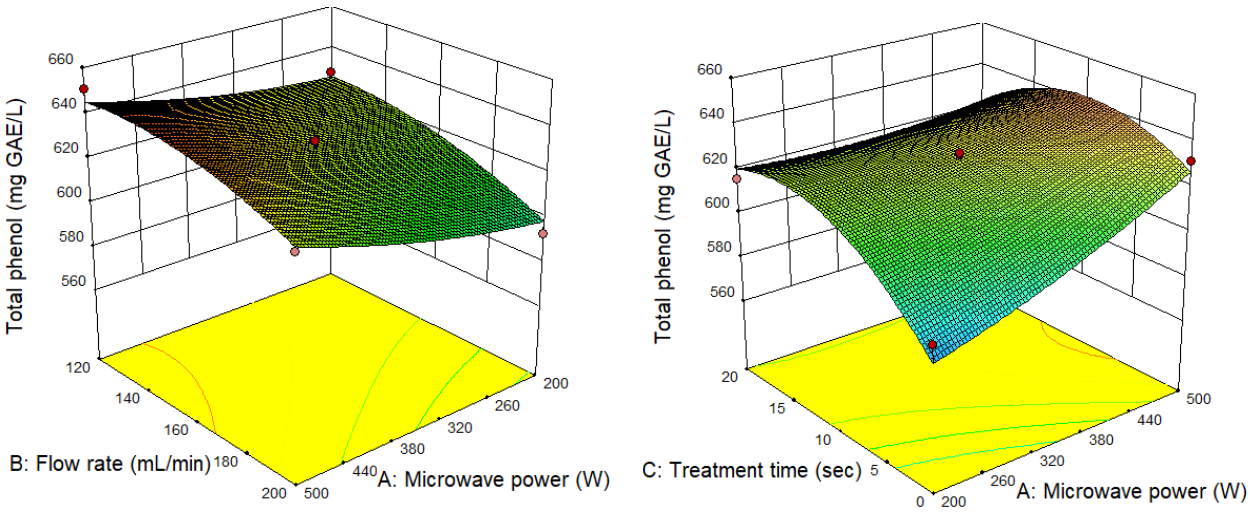

**(h)**

**Figure 2 (c-h).** Response surface plots (3D) for colour values (L*, a*, b*) as function of microwave power, flow rate and treatment time.




**(i)** **(j)**

**Figure 3 (i, j).** Response surface plots (3D) for vitamin C as function of microwave power, flow rate and treatment time.




**(k)** **(l)**

**Figure 4 (k, l).** Effect of microwave power, flow rate and treatment time on total phenolic content.

**Table 5.** Optimized values obtained from the Design expert software after optimization

| Variables | MWUV treated sample (Optimized values) | Control sample (Fresh orange juice) |
|---|---|---|
| Microwave power (W) | 500.00 | - |
| Flow rate (mL/min) | 166.00 | - |
| Treatment time (sec) | 9.51 | - |
| Total phenols (mg GAE/L) | 641.00 | 540.00 |
| L* | 57.63 | 61.82 |
| a* | 6.37 | 6.22 |
| b* | 53.81 | 54.26 |
| Vitamin C (mg/L) | 264.20 | 330.52 |
| Total plate count (log CFU/mL) | 1.26 | 5.38 |

### 3.4. Total phenols

Treatment time has a higher impact than microwave power on reducing total phenolic content when both the parameters are increased Figure 4 (k, l). With respect to the total phenolic content of the treated samples, the three independent variables: microwave assisted UV power showed a positive significant difference at $p < 0.001$; flow rate showed a negative significant difference at $p < 0.001$ on total polyphenols and treatment time showed positive significant difference at $p < 0.05$. Also the combination of microwave assisted UV power and treatment time showed significant negative difference at $p < 0.05$ on total polyphenols while the interaction effect of flow rate and treatment time showed significant positive difference at $p < 0.10$. The coefficient of determination ($R^2$) and adjusted coefficient of determination (Adj $R^2$) for the total polyphenols obtained were 90.75 and 78.85 (Table 3); hence the equation fits precisely well to the data points. Overall, there is increased in total phenolic content in the juice after the treatment with the increased in microwave power, flow rate and treatment time. Many researchers have also found the increased in phenolic content after heat or for the liquid foods like fruit and vegetable juices. radiation treatment of plant materials (Xu et al., 2007; Jeong et al., 2004; Gulati et al., 2003).

## 4. Conclusions

The optimum conditions for micrawave assisted UV sterilization system were calculated on the basis of microbial count (Total plate count), colour values (L*, a*, b*), total phenolic contents and vitamin C content. As per the design, the optimised independent parameters obtained were 500 W microwave power, 166 mL/min flow rate and 9.51 sec treatment time. Also, at the optimised condition Table 5, the values for thedependent parameters were total phenol: 621 mg GAE/L; L*: 57.63; a*: 6.37; b*: 53.81; vitamin C: 264.2 mg/L and total plat count: 6.46 log (CFU/mL). The system developed for the sterilization of liquid foods using microwave assisted UV treatment gave better results compared to microwave treatment alone in terms of microbial load and preservation of biochemical properties of foods with minimal treatment time. After a proper optimization of processing parameters, this system can be effectively scaled up to industrial and commercial level

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# THE EFFECTS ON CONTAGIOUS MASTITIS PATHOGENS IN BULK TANK MILK ON PHYSICOCHEMICAL PROPERTIES OF IRANIAN WHITE CHEESE

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**ABSTRACT**

The objective of this experiment was to study contagious mastitis pathogen effects on Iranian white cheese properties. Iranian white brine cheeses (2 trials) were made by healthy and containing pathogen bulk tank milk. All types of cheese were ripened at 13 °C for 2 weeks and at 6 °C to the end of ripening period. Cheeses were analyzed for the composition, color, lipolysis and proteolysis. The cheese of each trial was sampled at 1 and 60 days during ripening. No significant differences were observed between the fat, protein, ash and moisture contents of manufactured cheeses. Color parameters, lipolysis and proteolysis indices were not significantly different in two cheese groups. Therefore, incidence of *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacteria spp* and *Mycoplasma spp* in bulk milk tank does not change the physicochemical properties of Iranian white cheese. Despite the potential of these pathogens to cause health problems for consumers, they do not alter the characteristics of the cheese.

## 1. Introduction

Milk nature as complex biological liquid provides an excellent growth medium for many micro-organisms. It is unavoidable of milk contamination with micro-organism during its production procedure. So, the total microbial count of raw milk indicates the rate of its quality (Robinson, 2005). The source of bacterial contamination of raw milk can categorize into: air, milking equipment, feed, soil, feces and grass. Many factors can effect on the number and types of micro-organisms in milk immediately after milking such as animal and equipment cleanliness, season, feed and animal health (Coorevits et al., 2008). Quality of milk is deteriorated by the presence of subclinical mastitis and reduces milk production. Mastitis definition refers to inflammatory reaction of mammary tissues towards an infection; this inflammation is characterized by an influx of white blood cells into the mammary gland, followed by an increase in endogenous milk proteases. Mastitis causes economic losses for dairy farmers due to reduced milk yields (Heringstad et al., 2003) and it is the most expensive common diseases on dairy farm. Understanding the prevalence and

distribution of mastitis is a basic key to the prevention of disease. Microbial culture from bulk tank milk can be used as monitoring tool in the control and evaluation of clinical and subclinical mastitis. The most prevalent pathogens causing mastitis are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacteria spp*. and *Mycoplasma spp*.

Microbial methods for detection and numbering of specific micro-organism is an critical part of quality control and quality assurance plan and it can be used for raw materials, intermediate samples, finished products or environmental/equipment sites. Surveillance system of several reported that about Milk borne and milk-product borne outbreaks is 2-6% of food-borne outbreaks (De Buyser et al., 2001). One dairy product, which is so popular among Iranian people, is Iranian White cheese. This product is close-textured brined cheese, being similar to Beyaz Peynir (Turkish White cheese) and Feta but the way of Feta making procedure is different from Iranian White cheese. In Iranian cheese production, the period of dry salting of curd and slime formation on the curd surface before brining, which is essential for the development of the characteristic Feta flavor during ripening, does not exist (Sabbagh et al., 2010). Iranian cheese milk originated from cow's milk, sheep's milk or mixtures of them and the main flavor of it created due to acidity and saltiness (Khosrowshahi et al., 2006).

The objective of the present study was to study the composition and physicochemical properties of manufactured Iranian White cheeses from bulk tank milk containing contagious mastitis pathogens.

## 2. Materials and methods

### 2.1. Milk sample collection

A total of 30 bulk tank milk samples were collected randomly from industrial dairy herds of Fars, IRAN in 2011. First, the clean and dry teat was scrubbed with cotton soaked twice in 70% ethyl ethanol and the first squirt of milk was discarded. All collected samples were immediately put in an insulated container with ice pack and transferred to the laboratory without delay to perform bacterial culturing, PCR assay and to detect physiochemical parameters. According to the PCR results, samples were divided into healthy and contaminated group. Then, the mentioned assays were done on produced white brine cheese (Iran, 1999) from 2 groups on zero and 60- day.

### 2.2. Bacteriological culture

Milk samples were brought to room temperature and the mentioned microorganisms causing mastitis were isolated and identified according to previous methods reported by Carter and Cole (2012).

### 2.3. DNA extraction

DNA extraction was performed using a Cinnapur DNA kit (Cinagen, Iran). The specimens were centrifuged at 12000 rpm for 10 min. The supernatant was discarded and the pellet was vortexed and transferred into a 1.5 ml microtube. 200 µl of lysis buffer and 40 µl of proteinase K were added and incubated at 65 °C for 15 min. The DNA was further purified and re-suspended in 30 µl elution buffer according to the manufacturer's instruction, and kept at -20°C for further use. The concentration of DNA was subsequently estimated by absorbance at 260 nm and the purity of the DNA was checked by taking the ratio of O.D. reading at 260 and 280 nm using a spectrophotometer.

### 2.4. PCR assay

Four pairs of primers were used as previously described: species specific 225 bp, fragments 220-230 bp, 650 bp and 300 bp. which were subjected to *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacteria spp* and *Mycoplasma spp*.

respectively (Table 1). Multiplex PCR was carried out on 5 µl of the DNA template in a final reaction mixture of 50 µl containing 10 × PCR buffer, 1.5 mM MgCl2 (50 mM), 0.2 mM dNTP (10 mM), 1 µM of each of forward and reverse primer, 2.5 U Taq DNA polymerase (5 U/µl) (Cinna Gen, Iran). PCR cycling was performed in a gradient thermocycler (Eppendorf, Germany) with an initial denaturation step of 95°C for 5 min followed by 30 PCR cycles under the following conditions: denaturation at 95°C for 30s, annealing at 60°C for 30s, and extension at 72°C for 30s. After the final cycle, the preparation was kept at 72°C for 5 min to complete the reaction. (BIOR XP, China) (Jin et al., 2009). The amplified products were subsequently electrophoresed in a 2% agarose gel, stained with ethidium bromide and photographed under UV light.

Species specific fragments of 225, 220-230, 650 and 300 bp (Figure 1), corresponding to *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacteria spp* and *Mycoplasma spp*. were then amplified. Seven of these samples were used, and three negative samples for cheese making. For each trial, 3 kg of milk was used. Different types of cheese were produced according to the national standard for white brine cheese (Iran, 1999). Each types of the produced cheeses were ripened at 13 °C for 2 weeks and at 6 °C to the end of ripening period (60 days). Cheese of each trial was sampled at 1 and 60 days during ripening. All experiments on milk and cheese samples were done in triplicate.

### 2.5. Chemical analysis of milk and cheese

Titrable acidity of milk was determined by Dornic method. Digital milk scan (Lactostar, Funke Gerber, 230V) was used to determine fat, protein, solid nonfat, lactose and freezing point. The pH of milk and cheese samples was measured using a digital pH-meter (CG 824, Germany). Cheese was analyzed for moisture and dry matter content by vacuum-oven. Salt content was determined by Volhard method and fat content by the Gerber method. The ash content of cheese samples was determined by dry ash method and total protein content was determined by measuring total nitrogen using Kjeldahl method and converting it to protein content by multiplying by 6.38 (Sabbagh et al., 2010).

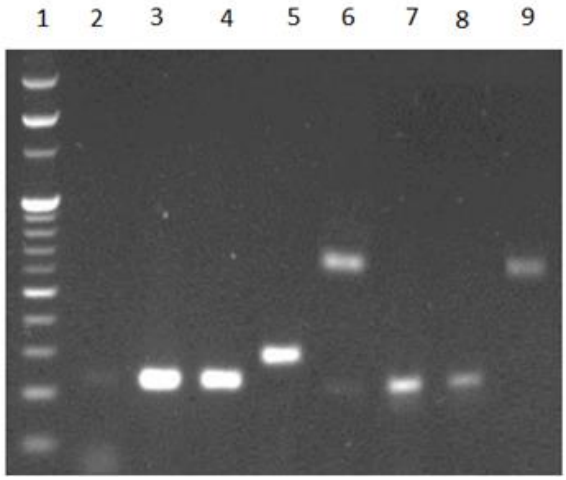


**Figure 1.** Representing 2% agarose gel electrophoresis for detection of *Staphylococcus aurous, Streptococcus agalactiae, Corynebacteria* spp and *Mycoplasma* spp, using multiplex PCR assay. Lane 1: 100 bp ladder, Lane 2: Negative control, Lane 3: 225 bp positive control, Lane 4: 220-230 bp positive control, Lane 5: 300 bp positive control, and Lane 6: 600 bp positive control, Lane 7: positive sample, Lane 8: positive sample, Lane 9: positive sample.

### 2.6. Cheese color measurement

The color of cheese samples at days 1 and 60 of ripening period was quantitatively determined using a simple digital imaging method described by Yam and Papadakis (2004), with a slight modification. Color values L*, a* and b* were determined.

### 2.7. Lipolysis

The acid degree value ADV was determined with a modified procedure developed by Park and Lee (2006).

### 2.8. Proteolysis

Water-soluble nitrogen (WSN) was determined in aliquots of water-soluble cheese extract (WSE) prepared as described by Fox et al., (2004). Total nitrogen (TN) of cheese samples and water-soluble nitrogen (WSN) of extracts were determined using the micro-kjeldahl method with an automatic digester model 430 and distillation unit model 322 (Buchi, flawil, hysic land). Index of maturation (IM) or Nitrogen Solubility Index (NSI) was expressed as a percentage of WSN of the cheese TN (WSN×100/TN) and it was used to follow the proteolysis degree during ripening. Analysis of the free amino groups in the WSE of the cheeses was determined according to the method of Fox et al., (2004). Data were analyzed by independent t test and Mann-Whitney U test (SPSS software, version 16, $p<0.05$).

**Table 1.** Primers that used for specification of gap gene, 16s–23s rRNA, 16s rRNA, deoxyribodipyrimidine photolyase (UVrC) gene amplicons corresponding to *Staphylococcus aurous*, *Streptococcus agalactiae*, *Corynebacteria spp* and *Mycoplasma* spp.

| Primers | Oligonucleotide sequence (5' 3') | Product size (bp) |
|---|---|---|
| **Au-F**<br>**Au-R** | TTCGTACCAGCCAGAGGT<br>TTCAGCGCATCACCAAT | 225 |
| **SU-F2**<br>**SU-R** | AGCCGCCTA AGGTGGGAT<br>ATGGAGCCTAGCGGGATC | 220–230 |
| **Cb-F2**<br>**Cb-R3** | CGTGCTTTAGTGTGTGCG<br>GGCACGGAAATCGTGGAAG | 650 |
| **Mb-F**<br>**Mb-F** | GCTTCAGTATTTTGACGG<br>GGTTTAGCTCCA TAACCAGA | 300 |

## 3. Results and discussions

### 3.1. Microbial and PCR analysis

Thirty raw bulk tank milk and their produced cheese samples were analyzed by means of both classic microbiological and molecular techniques, to detect *Staphylococcus aureus, Streptococcus agalactiae, Corynebacteria spp.* and *Mycoplasma spp.* In agreement with microbiological tests: 5 milk samples were contaminated with Staphylococcus aurous and Mycoplasma bovis, 2 samples with *Streptococcus agalactiae* and one with *Corynebacterium bovis*.

PCR results of the various cheeses in day zero indicated that 3 samples were contaminated with *Staphylococcus aurous*, and 2 samples with *Mycoplasma bovis*. After 60 days of cheese ripening, pathogen contaminations in cheese samples were reduced and *Staphylococcus aurous* was detected in one sample. So, *Staphylococcus aurous* and *Mycoplasma bovis* have been transferred from contaminated milk to zero-day cheese. During cheese ripening, *Mycoplasma bovis* was destroyed, but *Staphylococcus aurous* was shown to be resistant to ripening conditions.

Microbial culture from bulk milk samples can be an effective tool for milk quality evaluation compared with the individual cow milk samples collection and examination, and it may be a useful method for indicating prevalence level of contagious mastitis pathogens in herd. The contagious pathogens settling in cow's udder are *Staphylococcus aureus, Streptococcus agalactiae,* and *Mycoplasma spp*; therefore, the presence of these mastitis causing organisms in bulk milk are strong indicator of

the occurrence of intramammary infections in the herd (Fox et al., 2005). In this research, *Staphylococcus aurous* was detected from 5 bulk tank milk samples, *Mycoplasma bovis*, *Streptococcus agalactiae* from 2 samples and *Corynebacterium bovis* from one sample. There is some concern that bacteria in raw milk may transfer to the raw dairy products and raise a risk of food poisoning to consumers (Headrick et al., 1998). As regarding the mentioned information, some samples of white Iranian cheese were positive as *Staphylococcus aurous* and *Mycoplasma bovis* in the first day of ripening, but *Mycoplasma bovis* was not diagnosed 60 days later.

In present study, *Staphylococcus aureus* was detected from the primary sampling of bulk tank milk and cheese at various stages of production. Jørgensen et al., (2005) isolated *Staphylococcus aureus* from 10 of 11 cows, and the farmer, equipment, the environment, and the cheese and *Staphylococcus aureus* concentrations in the cheese were not at significant level as a risk of staphylococcal food poisoning. Active lactic starter cultures in cheese usually inhibit *Staphylococcus aureus* growth. Lactic acid as a weak organic acid produced in the fermentation period of dairy products, and can interfere in pH homeostasis of bacteria resulting in stressed cells. In the undissociated form lipophilic form of lactic acid and other weak organic acids can freely diffuse across the bacterial cell membrane. Then, the acid can dissociate and release protons inside of cell and finally acidify the cytoplasm. Cell energy is used to maintain internal pH, and hence, cell growth is reduced or inhibited, but in our study, *Staphylococcus aureus* was detected in 60 day of cheese maturation. The reason can be that *Staphylococcus aureus* is one of the most halotolerant, non-halophilic bacteria, and can grow in the presence of up to 3.5 M NaCl. It has been proofed that high concentrations of NaCl inhibit growth (Armstrong-Buisseret et al., 1995).

In agreement with some researches, *Staphylococcus aureus* detection has been reported from cheese and raw milk. The prevalence of 20 to 38% *Staphylococcus aureus* in Norwegian raw milk products has been mentioned (Jørgensen et al., 2005). In a Swedish study, coagulase-positive *staphylococci* were detected in 38% of on farm manufactured raw goat cheeses (Armstrong-Buisseret et al., 1995). In France, *Staphylococcus aureus* is reported to be the most frequent cause of foodborne disease from raw milk cheeses (De Buyser et al., 2001) and in Norway, outbreaks of *Staphylococcus aureus* food poisoning have been attributed to raw goat cheese (Watkinson et al., 2001), raw cow cheese and potato-mash made with raw milk. According to the PCR and microbiological tests in our study, 16.6% bulk samples were contaminated to *Staphylococcus aureus.* In favorable conditions, *Staphylococcus aureus* may grow and produce enterotoxins in foods, and because the toxins are stable with respect to heat and storage they may be present in foods where viable *Staphylococcus aureus* are absent. Dairy animals are probably the main source of contamination of raw milk with *Staphylococcus aureus* (Vautor et al., 2003). In particular, dairy animals with subclinical *Staphylococcus aureus* mastitis may shed large numbers of *Staphylococcus aureus* into the milk. Jørgensen et al., (2005) reported that the bacteria were spread with the milk and product material to the equipment and the environment during milking and cheese production (Jørgensen et al., 2005). The sanitation process seemed effective in removal of *Staphylococcus aureus*.

*Mycoplasma bovis* as a causative agent of mastitis is responsible for considerable economic losses to the dairy industry. Because of the highly virulent and

pathogenic nature of *Mycoplasma bovis*, it is so vital to identify the infected animals at early stage of infection and cause lower overall impact on the herd. *Mycoplasma bovis* is an important mastitis agent that was detected in bulk tank milks and first day of cheese ripening in our study. It appears ripening situation inhibit mycoplasma growth in the 60 day of cheese ripening. It was found that osmotic lysis of Mycoplasma organisms depend on the temperature of incubation and the pH of the suspending medium, being lowest at pH values near neutrality. The organisms were resistant to osmotic shock at 0 °C, but lysed rapidly at 37 °C (Feenstra et al., 1991).

*Streptococcus agalactiae* and *Corynebacterium bovis* were noticeable in bulk tank milk, but they were not detected in the various times of cheese production. *Streptococcus agalactiae* infection in dairy cattle has a considerable role in reduction of milk quality and milk products. Milk from cows with subclinical mastitis decreases the quality of cheese and other manufactured milk products (Politis and Ng-Kwai-Hang, 1988).

According to EU Regulations (Regulation 853, 2004) the total mean of microorganisms should not exceed 100.000 per ml of raw cow's milk after production. In this study we aimed to investigate the microbiological quality of 30 bulk milk samples. We determined the total bacterial count higher than 100.000 cfu/ml in both healthy and contaminated tested sample, while Aaku et al., (2004) calculated $5.5\times10^6$ cfu/ml of the total mean of micro-organisms in pooled raw milk, which is higher than in our study.

Coliform counts higher than100 cfu/ml are regarded by some authorities as unfavorable hygiene production. High coliform counts may be attributed to unrecognized coliform mastitis, mostly by *E. coli*. The coliform micro-organisms are also found on the surface of the under washed or moisture milking equipment.

In present study, coliform count and total bacterial count in contagious mastitis group were higher than healthy group.

### 3.2. Compositional and physicochemical properties

The milk and cheese compositional characteristics are shown in Tables 2 and 3 and two groups do not show any significant differences. The fat and protein contents of manufactured cheeses decreased significantly during ripening, while the ash, moisture, salt and pH of cheese increased ($p<0.05$).

**Table 2.** Chemical and bacteriological analysis of bulk tank milks that used for cheese making (Mean±SD)

| Composition | Fat (%) | Protein (%) | Lactose (%) | SNF [a] (%) | FPP [b] (%) | pH (%) | Acidity (%) | Coliform count (log cfu $ml^{-1}$) | Total count (log cfu $ml^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| A[c] | 2.49 ± 0.80 | 3.09± 0.27 | 4.77 ± 0.89 | 8.84 ± 0.64 | -0.49 ± 0.06 | 6.79 ± 0.16 | 18.48 ± 0.17 | 4.21 ± 0.27 | 6.11 ± 2.29 |
| B [d] | 2.62 ± 0.86 | 3.22 ± 0.23 | 4.89 ± 0.42 | 9.02 ± 0.69 | -0.51 ± 0.06 | 6.67 ± 0.15 | 18.03 ± 0.14 | 4.87 ± 0.31 | 6.32 ± 3.17 |

[a] SNF= solid non fat; [b]FPP= freezing point
[c] A= healthy milk; [d]B= milk containing pathogens

**Table 3.** Composition of Iranian white cheese in brine during the 60 days storage (Mean±SD)

| Treatment | Day | Fat (%) | Protein (%) | Ash | Moisture | Salt | pH CH | pH WH |
|---|---|---|---|---|---|---|---|---|
| **A** [a] | 0 | 18.50 ± 4..25 | 16.95 ± 1.32 | 3.11± 0.38 | 48.29± 2.45 | 2.81± 0.25 | 5.49± 0.25 | 6.17± 0.48 |
| **A** | 60 | 10.22± 3.51* | 13.5± 1.65* | 3.89± 0.28* | 53.56± 2.74* | 3.70± 0.33* | 5.98± 0.18* | 6.34± 0.41 |
| **B**[b] | 0 | 17.75±3.16 | 15.37± 1.04 | 3.09± 0.30 | 47.77± 1.95 | 2.69± 0.34 | 5.35± 0.11 | 5.97± 0.35 |
| **B** | 60 | 10.80±2.80 * | 12.47± 1.44* | 3.64± 0.15* | 53.19± 3.12* | 3.52± 0.27* | 5.85± 0.28* | 6.20± 0.29 |

*indicates $p<0.05$ between different days of each group

[a]A= cheese made with healthy milk [b]B= cheese made with milk containing pathogen

A slight increase in fat, lactose of milk was seen from healthy animal to infected groups, but no significant decrease was observed in protein, SNF, pH of milk. The exact composition of milk varies with the breed, species, feeding regimes and udder health (Ahmad et al., 2007). Subclinical mastitis changes the milk composition and any variety in its percentage affects the suitability of milk processing and the quality of its products (Sharif and Muhammad, 2009).

The degree of these changes depends on infecting agent and the inflammatory response. In present study, fat percentage in cow's increases from (2.49 ± 0.80) in milk from uninfected cows to (2.62 ± 0.86) in milk from infected cows, these results are in agreement with Schmitz et al. (2004), but disagree with Lehloenya et al., (2008). Some research reported that milk from infected animals with sub- clinical mastitis had significant increase in the activity enzyme called lipase that causes milk fat breakdown (Uallah et al., 2005), which is not compatible with our result about fat percentage. Also, cow's protein content was lower in milk from infected animals than in uninfected animals. These results were in agreement with Lehloenya et al., (2008), though disagree with Ullah et al., (2005). In this study, it appeared that solid nonfat (SNF) in infected animals was higher than SNF in healthy cows. The results are in accordance with the finding of Merin et al., (2004) in cows but disagreed with Hussain et al., (2012). Lactose content indicates a slight increase which supports Hussain et al., (2012) and is not in agreement with Dhillon et al., (2000). The pH was lower in the infected animals' milk. pH results proved (Hussain et al., 2012) and do not underscore (Ogola et al., 2007).

In this study as mentioned above, cheese was stored for 60 days at 6 °C. Madadlou et al., (2006) reported that Iranian white cheese in brine at 5 °C after 50 days changes chemically, biochemically and in opacity. As ripening progressed, moisture and protein contents continuously decreased, whereas their total ash and salt increased. Fat content and pH of cheeses remained stable during ripening (Madadlou et al., 2006). The results of that study about decreasing of moisture and protein content are in agreement with our results, although unchanging lipid and pH of cheese contradicts our findings, as in our study the fat decreased, whereas the level of pH increased. The results of Navidghasemizad et al., (2009) research indicated that the main cause for pH decrease in cheese is starters due to slow growth and activity of starter's lactic acid bacteria of

cheese that made from milk of cattle with mastitis, which are in competition with bacteria in milk, the level of pH increased, probably due to removal starters with the whey.

In our study, moisture and protein contents decreased (Table 3), while ash and salt increased in two groups. It possibly explained by net movement of NaCl molecules from the brine into the cheese texture as a result of the osmotic pressure difference between the cheese moisture and the brine. Consequently, the water in the cheese including dissolved materials such as acids and minerals spread out through the cheese matrix with a flux approximately twice that of NaCl so as to obtain osmotic pressure equilibrium (Guinee et al., 2002), and this decreased the moisture content and increased their salt content of samples as ripening progressed. Lower pH at renneting period decrease the net charge on casein micelles and probably improved the activity of rennet (Guinee et al., 2002), going to greater protein recovery in the curd. This, therefore, increased the protein content of cheese treatments. Banks et al., (1987) proved that the amount of CP in cheese from normally heat-treated (72°C for 16 s) and acidified milk (pH 5.8) was higher than unacidified milk. In the present study as shown in Table 2 and 3, the high level of pH could have an adverse effect on protein content.

### 3.3. Cheese opacity

The L*, a* and b* values of manufactured cheeses are presented in Table 4. L* and b* values of samples do not differ significantly ($p>0.05$). Ripening time change L*, a* and b* values of cheese treatments, such that L* value decreased during the ripening period, but a* and b* values increased ($p<0.05$).

Regarding the scattering of light by any about opacity, it should be noted that system is related to its heterogeneity (Pastorino et al., 2002). In a solid texture of dairy products such as cheese, light can cross over the superficial layers and is scattered by milk fat globules (Lemay et al., 1994) and whey pockets (Paulson et al., 1998).

**Table 4.** Instrumental color analysis (mean ± S.D) on the surface of the studied cheeses at days of 0 and 60.

| Treatment | Day | $L^*$ | $a^*$ | $b^*$ |
|---|---|---|---|---|
| **A** | 0 | $83.25 \pm 1.33^a$ | $-1.33 \pm 0.71^a$ | $1.72 \pm 0.79^a$ |
| **A** | 60 | $75.78 \pm 1.65^b$ | $-2.33 \pm 0.83^b$ | $13.5 \pm 1.15^b$ |
| **B** | 0 | $83.09 \pm 1.57^a$ | $-1.84 \pm 0.41^a$ | $1.78 \pm 0.39^a$ |
| **B** | 60 | $74.35 \pm 2.44^b$ | $-2.67 \pm 0.95^b$ | $14.25 \pm 1.41^b$ |

A= cheese made with healthy milk B= cheese made with milk containing pathogen
L*; 0 = black and 100 = white; a*; -60 = green and +60 = red; b*; -60 = blue and +60 = yellow.
In each treatment, means within the same column with different superscript letters differ significantly ($p<0.05$).

In this experiment, whey transferred from cheese texture into the brine as ripening progressed and finally cheese moisture decreased (Table 3). The surface area

occupied by light-scattering centers was therefore decreased. Kaya (2002) reported that the ripening of Gaziantep cheese in the weakest brine caused highest moisture content and the highest L value compared with samples ripened in stronger brines. Color changes due to starter concentration were happened spontaneously to changes in the moisture content of the treatments at a given ripening time, which end in lower light scattering and L values.

### 3.4. Lipolysis

The ADV of cheeses are presented in the Table 5 and no significant differences are observed between two groups. The acid degree value of cheeses increased significantly during ripening period.

### 3.5. Proteolysis

High SCC milk usage also negatively influence on flavor, body and texture grades. For example, cheddar cheeses from high SCC milk samples have been indicated a "lipolytic" or "oxidized" flavor and milk for cheddar cheese production have been presented a higher concentration of free fatty acids, which can cause rancidity in dairy products. It has been reported that the flavor or texture defects in mozzarella, Prato or ewes cheeses made with high SCC milk, is due to higher levels of lipolysis or proteolysis in the cheese (Andreatta et al., 2007). These mentioned results were compatible with our data about acid degree value of cheeses which increased significantly during ripening period.

The TN, WSN, NSI and FAAs of cheeses did not show any significant difference between two groups (Table 5). TN and WSN of cheeses decreased significantly during ripening and NSI increased ($p<0.05$). FAA contents showed a non-significant increase during ripening period.

Cheese proteolysis in ripening period is the concerted action of proteolytic enzymes. At the initial stage of ripening, enzymes such as chymosin and plasmin (an indigenous proteinase in milk) affect intact casein in the cheese curd.

Proteinases and peptidases of lactic acid bacteria cause further breakdown of casein, large peptides, and oligopeptides into small peptides and amino acid and transfer to the secondary stage of ripening (Attaie, 2005).

**Table 5.** Acid degree value, total nitrogen (TN), water soluble nitrogen (WSN), nitrogen solubility index (NSI) and free amino acids (FAAs) of the studied Iranian white cheese in brine.

| Treatment | Day | Acid degree value | TN% | WSN% | NSI | FAA(Ab 507 nm) |
|---|---|---|---|---|---|---|
| **A[a]** | 0 | 1.32±0.28 | 3.38±0.40 | 0.05±0.01 | 1.59±0.50 | 1.46±0.81 |
| **A** | 60 | 2.43±0.51* | 2.11±0.31* | 0.14±0.06* | 6.53±2.79* | 1.67±0.64 |
| **B[b]** | 0 | 1.47±0.32 | 3.25±0.51 | 0.06±0.02 | 1.63±0.82 | 1.42±0.53 |
| **B** | 60 | 2.78±0.48* | 2.14±0.39* | 0.14±0.07* | 7.11±3.49* | 1.63±0.56 |

*indicates $p<0.05$ between different days of each group

aA= cheese made with healthy milk B= cheese made with milk containing pathogen.

These changes cause flavor development through the release of free amino acid and texture (Sabbagh et al., 2010), which will increase the pH level. Primary proteolysis of caseins in mastitis milk brings about proteolysis increase of β-caseins during the

early stages of cheese ripening. Also, it causes an accelerated breakdown of $\alpha S_1$-casein. As a consequence, protein losses in the whey are increased. In present study, a non-significant increase of FAA, a significant decrease of TN and WSN and significant increase of NSI were observed. In other research, cheese made with high SCC milk samples have been shown higher levels of proteolysis regardless the cheese type (Le Maréchal et al., 2011).

As Table 5, the FAAs in both the control and treatment (A, B) were increased after 60 days, and the amount of FAAs in control group was higher than treatment as a result of free AA movement into the brine. As the starter concentration in milk increased, the amount of free tyrosine-tryptophan in cheese increased at a known storage time. The strong explanation for starter cells increase retained at the curd is the higher initial cells used to inoculate the milk or pH decrease at wheeling or both and caused more production of proteinases and peptidases that were excreted into curds and resulted in more breakdown of casein molecules (Attaie, 2005) and larger peptides to smaller peptides and free AA. The lower pH values of the treatments with higher starter may lead to more hydrolysis of casein molecules. However, it may slightly improve the activity of retained rennet at the curd (Watkinson et al., 2001).

## 4. Conclusions

In this research the results didn't show any difference between physicochemical properties of the manufactured cheeses. The reason of this result is high total bacterial count in milk of Iranian dairy farms. Therefore, In Iran, the incidence of contagious mastitis pathogen is important for safety of dairy products and no for quality of them.

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# TRACE METAL ANALYSIS OF ORGANIC VEGETABLES SOLD IN SOME SUPERMARKETS IN MANILA, PHILIPPINES

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**ABSTRACT**

Recent years have seen the rapid growth of the *organic* food products industry, primarily driven by the consumers' desire for a healthier lifestyle. Similar to worldwide trend, explicitly-labeled "Organic" food products have become ubiquitous in the Philippines, with the consumers most of the time having no information on the quality of the products. In the Philippine setting, very few researches have focused on the analysis of *organic* vegetables, and in this study, the trace-metal (cadmium, copper, iron, nickel, and zinc) concentration of specific organic and conventional vegetables (cabbage, celery, leek, lettuce, and spinach) that are being sold in some shops in the cities of Makati and Manila, Philippines were determined using atomic absorption spectroscopy. The mean concentration for copper, iron, nickel and zinc in the samples were calculated to be between 0.0146-0.881 mg/kg, 0.648-13.1 mg/kg, 0.0409-2.04 mg/kg, and 0.266-2.87 mg/kg, respectively, while cadmium levels varied from 0.005-0.772 mg/kg (with some samples below the limit of detection). Nevertheless, statistical analysis ($p < 0.05$) showed more organic vegetables having no significant differences than conventional ones indicating that in terms of the content of these trace metals, being organic may not necessarily mean better.

## 1. Introduction

Vegetables are very important when it comes to the eating habits of humans in order to be healthy. These food products provide many health benefits which include being a good source of nutrients such as folate or folic acid, fibers, potassium, vitamins A, E, and C, in addition to having antioxidant effects (Slavin, 2012). These nutrients found in vegetables are crucial and important in the health and well-being of the body, but they may also pose negative effects to a person's health due to potential occurrence of high concentration of certain metals. Research has shown that vegetables grown in contaminated farmlands absorb chemicals which cause heavy metal contamination of crops such as cadmium, iron, nickel, etc. (Wuana, 2011). These heavy metals cannot be processed by the body and can undergo bioaccumulation inside an organism.

Heavy metals can infiltrate the food cycle, vegetables in particular, through environmental contaminations such as the application of certain agricultural aids like fertilizers and pesticides to the soil or to the crop (Wuana et al., 2011). Crops that are grown without the use of these agricultural aids and instead are grown with renewable resources so as to improve its environmental quality and health benefits are called "organic",vand are reported to have lower levels of heavy metals.

Plants have diverse capabilities when it comes to the removal and the accumulation of metals (Chibuike, 2014). Vegetables that are leafy tend to absorb and accumulate more metals from its environment (Ali et al., 2012). There is even the possibility that metal contamination in vegetables may increase due to an escalation in the heavy metal concentrations of certain soils due to the rapid increase in industrial and urban activities. Nevertheless, consuming certain metal-contaminated vegetables (e.g.. high heavy metal content) may pose serious health risks to humans since these are not easily metabolized and thus can be bioaccumulated in certain vital organs, causing various types of sickness depending on the chronic and acute exposure (Chibuike et al., 2014).

The organic industry of the Philippines was valued at US $10 million during the year 2003, with an estimated 20% annual growth rate (Pearl2, 2007). In 2014, the Philippines had the fourth largest number of organic producers and most organic agricultural land at 165,974 producers and 101,278 hectares respectively among Asian countries (Willer, 2016). The consumption of vegetables in the Philippines has been increasing through the years from 1990-2005; the 1.6% increase in annual consumption of vegetables surpassed the 1.5% increase in annual vegetable production (Porciuncula, 2014). Unfortunately, reports on trace metal content of organic and conventional vegetables being sold in the Philippine market are lacking, and since buying organic produce usually costs higher, it is important for consumers to know if they are indeed getting their money's worth. In this study, trace metal content of different vegetables and brands, both organic and conventional, being sold specifically in Makati and Manila, were determined and analyzed to help serve as a guide for consumers in their decision-making process.

## 2. Materials and methods

### 2.1 Vegetables Samples

Three brands of organic vegetables were identified and purchased in supermarkets in Makati and Manila, Philippines. For each organic brand, five vegetables were bought, namely cabbage, celery, leek, lettuce, and spinach. For conventional samples, the same five vegetables were bought from side-road vendors.

### 2.2. Preparation of vegetable samples

For the duration of the experiment, only the leaves of the vegetables were used. The collected leaves were rinsed in distilled water and wrapped individually, separating one vegetable from another, with properly labelled aluminum foil. Rinsing and drying of samples were done in batches, based on the brand of vegetables, over the course of 3 weeks. The samples were dried in an oven at 100$^{o}$C to 120$^{o}$C, each batch requiring 3 - 4 hours for the drying process to be completed. After drying, the samples were then subjected to acid digestion (Hunt, 1982).

Two grams of each vegetable was accurately weighed using an analytical balance and transferred to 250 mL Erlenmeyer flask. 25 mL of 12 M HCl was added to each flask, followed by heating using the hot plate at low setting for twenty minutes under a hood. Afterwards, 10 mL of distilled water was added. The solution was filtered to 50 mL volumetric flask three times using filter papers. The filtrate was diluted to the mark with distilled water passing through the filter paper. The entire process was done in triplicates for each vegetable.

### 2.3. Trace Metal Analysis

The concentrations of copper, iron, nickel, and zinc in each of the digested samples were determined using the AA-6300 SHIMADZU atomic absorption spectrophotometer. A stock solution containing 100 ppm of all four metals to be analyzed was purchased from Thermo Fischer Orion. Varying amounts of the stock solution were transferred to 50 mL volumetric

flasks and diluted to the mark using distilled water to prepare standard solutions having concentrations ranging from 0 ppm to 15 ppm. The type of gas used was air-$C_2H_2$, with burner height of 7 mm and slid width of 0.7 nm. For all of the trials, the fuel gas flow rate was 1.8 L/min while the support gas flow rate was 15.0 L/min. A standard curve was also formulated for each of the metal analyzed. The wavelengths used for the analysis were 228.8 nm, 324.7 nm, 248.3 nm, 232.0 nm, and 213.9 nm for cadmium, copper, iron, nickel, and zinc respectively.

### 2.4. Statistical Analysis

The data gathered were compared and analyzed using one-way analysis of variance and Tukey multiple comparisons test ($p < 0.05$) using Graphpad Prism ver. 6.07.

## 3. Results and discussions

Despite their apparent necessity in human diet, studies have shown that vegetables can absorb and accumulate metals from the soil due to sewage effluents, waste water irrigation, conventional pesticides, and synthetic fertilizers (Alloway, 2013). Concerns on the health risks posed by excessive intake of trace metals, among others, have increased the consumers' demand for organic food products that are deemed to be the healthier option compared with conventionally grown produce. However, there is a lack of scientific evidence in support of this claim, especially in the Philippine market.

Using atomic absorption spectroscopy, samples of five organic and conventional vegetables (cabbage, celery, leek, lettuce, spinach) were analyzed for their trace metal (Cd, Cu, Fe, Ni, Zn) concentrations. In the experiment, metal content in the vegetables generally followed this trend: Fe > Zn > Ni > Cu > Cd (Figs. 1 and 2). These results were found to be in accordance with several studies reported earlier. In a study comparing Fe, Cu, Cd and other metal concentrations in 13 vegetable samples taken from agricultural sites in Punjab, India, Fe was accumulated the highest and Cd was accumulated the lowest for every vegetable (Sharma, 2016). Furthermore, a study done by Hoque et. al. concluded that among Zn, Cu, and Ni, the highest average concentration of trace metals in 8 samples (tomato, bottle gourd, green amaranth, red amaranth, chili, and banana ) was found to be Zn, while Ni had the lowest concentration among the three metals (Hoque, 2014). Another study done produced a similar trend with regards to trace metal concentration in spinach, lettuce, cabbage, coriander, radish, and cauliflower (Anwar, 2008). Other studies concerning the estimation of heavy metals in other vegetables reported similar results (Arora, 2008; Singh, 2010; Uwah, 2011).

Varying amounts of metals in vegetables can be attributed to a number of factors such as natural and anthropogenic sources, oxidation and reduction reactions, adsorption and dissolution reactions, and nonorganic and organic complexations of these trace metals in the soil. For Fe, its abundance is due to it being a biologically essential component of every living organism (Aisen, 2001; Lieu, 2001). In addition, sources of Fe in the soil are mineral compounds that can constitute about 30-40% of the earth's composition, deteriorating metals, and industrial wastes (Polanski, 1988). For these reasons, it is sensible that several studies recorded iron concentration to be among the highest in various vegetable samples. On the other hand, Cu accumulates mainly in the root system of the crops because under excessive concentration of Cu, root tissues of plants have shown a strong capability to hold Cu from being transported to the shoots (Bigdeli, 2008). Thus, only a small fraction of Cu will be found in the shoot system of the crop. Since this report only analyzed trace metal concentrations in the leaves, this may be a probable reason behind lower Cu concentration found in the samples compared to other trace metals. It was also remarked that Cu deficiency can be observed in soils that are rich in organic matter which reduce mobility and availability of Cu (Oorts, 2013). This is due to its tendency to be adsorbed by carbonates and clay minerals, both

of which having a rather large binding capacity for Cu (Logan, 1997). Compared to other

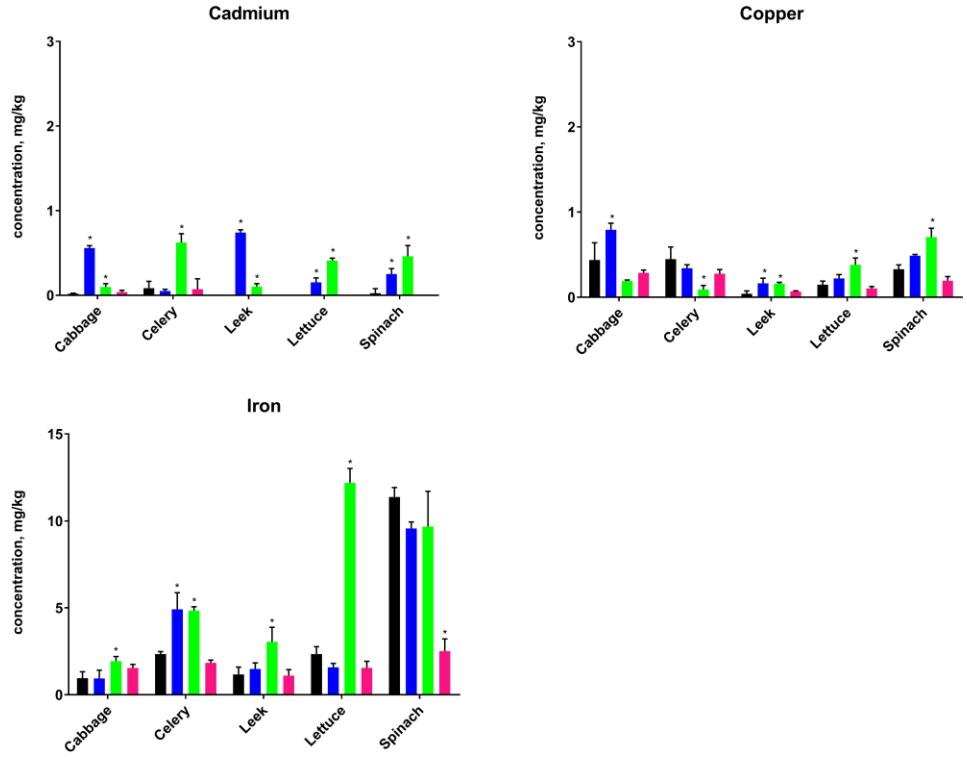


**Figure 1**. Average concentration of cadmium (Cd), copper (Cu), and iron (Fe) in various vegetables and brands. Black bars represent conventional vegetables, while blue, green, and pink bars represent Brand A, B, and C organic vegetables, respectively. Some samples have Cd concentrations that are below the limit of detection. Error bars indicate standard deviations. Asterisk indicate significant differences with the conventional vegetables ($p < 0.05$). The y-scale for Fe is different from the other metals.

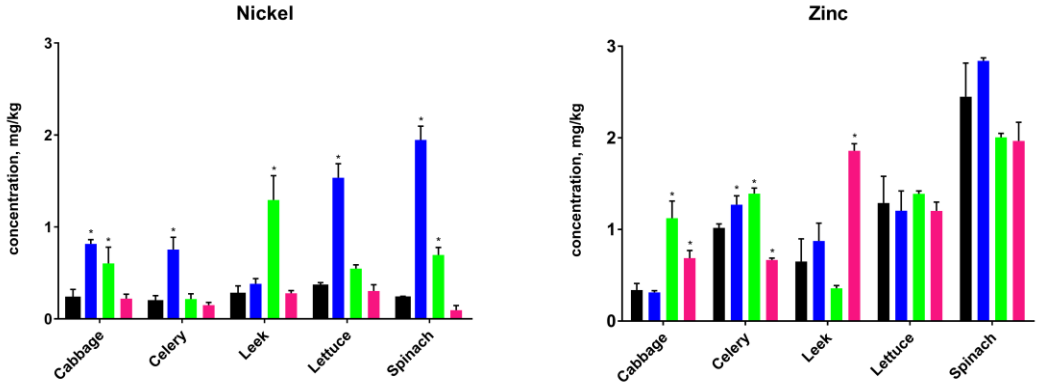


**Figure 2**. Average concentration of nickel (Ni) and zinc (Zn) in various vegetables and brands. Black bars represent conventional vegetables, while blue, green, and pink bars represent Brand A, B, and C organic vegetables, respectively. Error bars indicate standard deviations. Asterisk indicate significant differences with the conventional vegetables ($p < 0.05$).

metals, concentration of Cd in earth's crust is relatively low at 0.1 to 0.5 ppm (Jensen, 1992) and this may be linked to the low Cd concentration detected in the vegetable samples.

Despite the similar trend on metal concentration found in a variety of vegetables, it is important to note that the range of metal concentrations differ from each study. This difference can be attributed to several environmental factors surrounding vegetable growth and development. Several sources of contamination of the environment where vegetables are planted include emissions from the rapidly expanding industrial areas, disposal of wastes, animal manures, leaded gasoline and paints, land application of fertilizers, mine tailings, pesticides, wastewater irrigation, coal combustion residues, spillage of petrochemicals, and atmospheric deposition (Khan, 2014; Zhnag, 2014)

Furthermore, different vegetables have different characteristics that may enable them to accumulate more metals than others. In this report, spinach contained higher metal concentrations compared to other vegetables in all but a few cases (Figs. 1 and 2). Spinach has leaves with a large surface area that makes it more likely to accumulate metals (Ali, 2012). Spinach also has a higher transpiration, in which moisture is carried from the roots to the leaves of the plants to be released. Due to this and spinach's faster growth rate, translocation of metal in spinach is faster and has higher rate of metal absorption (Muchuweti, 2006; Alia, 2015). These findings are also consistent with previous literature on spinach and its capability to absorb and retain trace metal (Osaili, 2016; Hoefkens, 2010; Bvenura, 2012).

Data gathered in this report were subjected to statistical analysis and the presence or absence of significant differences among the different samples were determined using the Tukey multiple comparisons test at $p < 0.05$ (Figs. 1 and 2). Results show that trace metal content of majority of organic vegetable samples did not differ significantly from their organic counterparts, and among those that are significantly different, organic samples were found to have higher metal concentrations than their conventional counterpart except in a few cases. Other literatures comparing conventional and organic vegetables have likewise reported that conventional vegetables have less metal concentration than the organic ones. As an example, a report has concluded that contaminants in organic vegetable samples were slightly higher than the conventionally grown vegetables (Hoefkens, 2010). Furthermore, studies comparing nutrients in conventional and organic vegetables showed lack of evidence that the two populations have significantly different nutrients contents (Bourn, 2002; Dangour, 2009; Williams, 2002). While a trend in the metal concentration of vegetable samples is observable, most of the trace metals analyzed are essential nutrients for various biochemical and physiological functions. Their functions in biological systems are well-known; for example, trace metals can participate in redox reactions, and they can also bind to proteins in enzymatic systems, and deficiency, diseases or syndromes may be evident when trace metals are supplied inadequately. However, upon reaching a certain level, accumulation of these same trace metals may result to serious health concerns. Excess Fe was determined to cause organ dysfunction due to the production of reactive oxygen species (ROS) (Kohgo, 2008). Similarly, accumulation of Cu produces ROS that lead to aging and the development of cancer and diseases of the nervous system (Halliwell, 1990). At higher concentrations, Zn and Ni are also associated with reduced immune function, lower levels of high-density lipoproteins, decreased body weight, and infections in the intestine (Hamilton, 2000; Henderson, 2012). Unlike the rest, Cd is not involved in human nutrition and higher biological systems. It is classified as a carcinogenic and exposure to higher levels of Cd is associated with prostate, renal, and lung cancers (Jarup, 2002; Waalkes, 2003). The mean concentrations of heavy metals in various vegetable species were compared with the standards set for vegetables

by FAO & WHO (2014). These values are summarized in Table 1.

**Table 1**. Range of trace metal content (mg/kg) of various vegetables analyzed compared to safe limits established by WHO/FAO.

| | **Copper** | **Iron** | **Nickel** | **Zinc** | **Cadmium** |
|---|---|---|---|---|---|
| **Cabbage** | 0.182 – 0.881 | 0.648 – 2.24 | 0.106 – 0.867 | 0.266 – 1.33 | 0.0186 – 0.587 |
| **Celery** | 0.0391 – 0.605 | 1.74 – 5.98 | 0.106 – 0.908 | 0.649 – 1.46 | 0.00500 – 0.711 |
| **Leek** | 0.0146 – 0.231 | 0.716 – 3.91 | 0.156 – 1.60 | 0.330 – 1.92 | 0.0740[a] – 0.772 |
| **Lettuce** | 0.0852 – 0.464 | 1.17 – 13.1 | 0.235 – 1.71 | 0.954 – 1.49 | 0.119[a] – 0.442 |
| **Spinach** | 0.165 – 0.790 | 1.97 – 12.0 | 0.0409 – 2.04 | 1.77 – 2.87 | 0.0282[a] – 0.596 |
| **Tolerable Intake WHO/FAO*** | 0.5 mg/kg bw per day | 0.8 mg/kg bw per day | 1.2 mg/kg bw per day | 0.3-1 mg/kg bw per day | 25 μg/kg bw per month |

* bw: body weight

[a] some samples had Cd levels below the detection limit

## 4. Conclusions

This study reports the trace metals (Cd, Cu, Fe, Ni, and Zn) content of cabbage, celery, leek, lettuce, and spinach sold in some supermarkters in Manila, Philippines, both organic and conventionally-produced. The order of the metal content in the vegetables generally followed this trend: Fe > Zn > Ni > Cu > Cd. The mean concentration for Cu, Fe, Ni and Zn in the samples were calculated to be between 0.0146-0.881 ppm, 0.648-13.1 ppm, 0.0409-2.04 ppm, and 0.266-2.87 ppm, respectively, while cadmium levels varied from 0.005-0.772 mg/kg with some samples containing amounts less than the instrument detection limit. Discrepancies between each sample and its metal content can be attributed to several factors. One of which is the type of vegetable used. Spinach was shown to absorb the highest metal concentration. This may be attributed to physical characteristics of spinach, its higher metal translocation, and faster metal absorption rate. Statistical analysis ($p < 0.05$) showed more organic vegetables having no significant differences than conventional ones indicating that in terms of the content of these trace metals, being organic may not necessarily mean better. The results in this study may serve as a guide for consumers in their choice between organic and conventionally produced vegetables, although more analysis is needed to give a complete picture of the chemical contents of these two types of products.

## 5. References


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